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USE OF IRON SALTS FOR CONTROL OF ACTIVATED SLUDGE BULKING CAUSED BY *SPHAEROTILUS*

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F I N A L R E P O R T

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ABSTRACT

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A continuing operational problem encountered in the activated sludge system is sludge bulking. While there are several causitive agents for this bulking, the filamentous bacterium *Sphaerotilus* is one of the more common. Control of the growth of this organism will help to eliminate sludge bulking as an operating problem in many activated sludge systems. Iron has been identified in the literature as a possible inhibitor to the growth of this bacterium. However, little is known about the mechanism of this inhibition. This study has shown that the adsorption of iron on *Sphaerotilus* is the major inhibitory mechanism. The layer of iron on the organism appears to block the transport of nutrients through the sheath and cell wall and hence inhibit the growth of this organism. The effectiveness of the iron compounds in this inhibition corresponds to the physical characteristics of the absorbed iron. Soluble iron complexes form a uniform layer so that the inhibitory effect is proportional to the iron adsorbed. Among the soluble complexes, the ferrous forms are more effective. These forms can penetrate the sheath and deposit on or near the cell wall resulting in greater inhibition. On the other hand, the ferric complexes are deposited on or in the sheath of the organism.

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KEYWORDS: *activated sludge/*sludge bulking/*sphaerotilus inhibition/ *sewage treatment

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1. INTRODUCTION

The activated sludge process is the most common secondary wastewater treatment process used in the United States. One of the difficulties encountered in the operation of this process is the phenomenon of sludge bulking. Bulked sludge settles poorly and does not compact well in the secondary settling tank. Consequently, large quantities of the activated sludge solids remain in the effluent from this settling tank. These solids add substantial BOD to the secondary treatment plant effluent, or add a substantial solids and BOD load to any tertiary treatment process. If the sludge bulking is not corrected the process will eventually fail because of the inability to maintain an active population of microorganisms in the aeration tank. Prevention and control of sludge bulking is of considerable importance in the successful operation of the activated sludge process.

There are two types of sludge bulking, filamentous and nonfilamentous. The type of bulking can be distinguished on the basis of the morphological characteristics of the causative microorganisms. The nonfilamentous bulked sludge is characterized by a large quantity of bound water (Heukelekian and Weisberg, 1965), while filamentous sludge bulking is a result of the presence of a significant number of filamentous microorganisms. The bacterium *Sphaerotilus* is frequently associated with this condition, although other filamentous organisms may also be responsible for sludge bulking (Pipes, 1967a, 1967b; Farquhar and Boyle, 1971b).

Control of sludge bulking is a difficult problem. The success of measures taken depends upon the nature of the sludge. Whenever sludge bulking due to filamentous organisms has occurred in wastewater treatment

plants, the usual practice has been to add a large dose of chlorine to the return sludge (Smith and Purdy, 1936; Tapleshay, 1945). However, during the chlorination period the effluent usually becomes turbid and the BOD and suspended solids in the effluent increase.

Since *Sphaerotilus* growth has been reported to be inhibited by ferric iron (Waitz and Lackey, 1959), the addition of ferric iron to the bulked sludge may prove to be an effective method of controlling sludge bulking that is caused by this organism. The purpose of this study is to evaluate the feasibility of controlling the growth of *Sphaerotilus* with the addition of iron salts to the activated sludge system.

The specific objectives of this study are as follows:

1. To isolate and identify *Sphaerotilus* from a bulked activated sludge system.
2. To determine the dosage of iron required to control the growth of *Sphaerotilus*.
3. To ascertain the mechanism by which *Sphaerotilus* growth is controlled by iron.

II. *SPHAEROTILUS* AND SLUDGE BULKING

1. Filamentous Bulking

Filamentous bulked sludges result from the presence of a significant number of filamentous microorganisms. Filamentous bacteria or filamentous fungi may cause this condition to occur (Pipes, 1967a; Lackey and Wattie, 1940). Members of the following genera have been identified as causing bulking: *Sphaerotilus*, *Bacillus*, *Begiatoa*, *Nocardia*, *Thiothrix*, *Arthrobacter*, *Toxothrix*, *Vitrecoscilla* and *Geotrichum* (Pipes, 1967a, 1967b; Farquhar and Boyle, 1971b). Among these organisms, *Sphaerotilus*, *Toxothrix* and some species of *Thiothrix* are the sheath forming microorganisms (Farquhar and Boyle, 1971a).

Sphaerotilus isolated from bulked activated sludge grows well on the medium used by Stokes (1954) or that used by Lackey and Wattie (1940). When *Sphaerotilus* grows in a medium with low content of organic matter and high content of iron, it synthesizes a sheath outside its cell and iron may be found deposited on or in this sheath. In the presence of H_2S or Na_2S, *Sphaerotilus* may deposit sulfur inside the cell. However, the amount of sulfur in the *Sphaerotilus* cell is much less than that in *Thiothrix*. *Sphaerotilus* and *Toxothrix* both belong to the order of *Chlamydobacteriales* and they strongly resemble each other in morphology and cytology. However, *Toxothrix* has not been obtained in pure culture.

Many factors have been designated as contributing to the bulking of sludge and encouraging the growth of filamentous organisms; they are summarized as follows:

(1) Fluctuations in the concentration and volume of the waste (Sawyer, 1966).

(2) High or low food to microorganism ratio (Logan and Budd, 1955; Genetelli and Heukelekian, 1964; Ford and Eckenfelder, 1966).

(3) High concentration of carbohydrates in the waste (Lackey and Wattie, 1940; Morgan and Beck, 1939; Smit, 1934; Ingols and Heukelekian, 1940).

(4) Unbalanced nutrients in the waste, e.g., high BOD/N or BOD/P (Greenburg, *et al.*, 1955; Jones, 1965).

(5) Low pH (Jones, 1966; Pipes and Jones, 1963).

(6) Low levels of dissolved oxygen in the mixed liquor (Heukelekian, 1941; Haseltine, 1932).

(7) Stale sewage and septic return sludge (Heukelekian, 1941).

(8) High or low temperature (Ludzak, *et al.*, 1961; Heukelekian, 1941).

(9) Presence of H_2S in the waste (Farquhar and Boyle, 1971b; Sawyer, 1966; Waitz and Lackey, 1959).

(10) Types of reactors (Rensink, 1974).

(11) Deficiency of iron (Pfeffer, 1967; Carter and McKinney, 1973).

Sphaerotilus has for many years been the organism most commonly associated with filamentous bulking (Ingols and Heukelekian, 1939; Greeley, 1945; Ruchhoft and Watkins, 1929). Studies conducted in recent years by Jones (1964, 1965) and Pipes and Jones (1963) have revealed that *Geotrichum*, a member of the class *Fungi imperfecti*, may also cause filamentous bulking. In certain stages of growth the two filamentous organisms look considerably alike, and this, no doubt, is part of the reason why bulking caused by *Geotrichum* may have been credited to *Sphaerotilus* at various times.

In some of the above listed factors, the bulking of sludge could be more likely caused by fungi than *Sphaerotilus*. This is based on the

facts that fungi tolerate a wider range in pH and temperature than bacteria, and can grow under extreme nutrient conditions (Pelczar and Reid, 1972). Fungi grow over a pH range of 2.0 to 9.0 and the optimal pH for most species is 5.6. The optimal temperature for most species is 22 to 30°C, while some species will grow at 0°C and some grow at 62°C.

While the nitrogen content of bacteria is about 10 to 12 percent of the dry weight, fungi contain only 4 to 6 percent nitrogen (Jones, 1965; Kaylor, *et al.*, 1963). This gives fungi the competitive advantage for growth on a nitrogen deficient waste. The requirement of nitrogen and phosphate was found to be dependent upon the food to microorganism ratio (F/M). At a higher F/M, a larger percentage of substrate was used for cellular systhesis, and hence lower carbon to nitrogen and phosphate ratios are required for the growth of organisms (Walters, 1966).

The concept that low dissolved oxygen in the mixed liquor promotes the growth of filamentous organisms has been challenged by Bhatla (1967). His work with activated sludge treatment of kraft paper mill wastes indicated that his plant operated well in a filamentously bulked condition with 2.0 to 3.0 mg/l dissolved oxygen in the aeration tanks. The settleability of the sludge improved as the mixed liquor dissolved oxygen concentration was decreased. This discrepancy between the reports of the effect of oxygen concentrations on the sludge bulking can be explained as the difference between the fungus bulking and *Sphaerotilus* bulking. Although both *Sphaerotilus* and fungi are capable of metabolizing substrate at low oxygen tensions, the growth of fungi is stimulated by high oxygen concentration (Pelczar and Reid, 1972).

H_2S stimulation of the growth of *Sphaerotilus* was reported by several investigators (Farquhar and Boyle, 1971b; Sawyer, 1966; Waitz and Lackey, 1959). Farquhar and Boyle (1971b) related the sulfur containing species to *Thiothrix*; Waitz and Lackey (1959) suggested the classification of this organism as a sulfur bacterium. However, the autotrophy of this organism has not been demonstrated. In sewage treatment plants receiving sewage containing 0.3 - 0.5 mg/l of H_2S, bulking of sludge cuased by this sulfur depositing organism was observed (Farquhar and Boyle, 1971b). When the energy source is considered, this low concentration of H_2S is negligible compared with the high concentration of organic matter. Also, when sulfur dioxide was tested instead of hydrogen sulfide in the culture of Waitz and Lackey's species, the same deposition of sulfur occurred. After exposure, none of the organisms were viable (Waitz and Lackey, 1959). This observation showed that this organism did not use H_2S as food but was able to oxidize it. The role of H_2S in the wastewater may be considered as an indirect factor in stimulating the growth of *Sphaerotilus*. The toxicity of H_2S to other organisms provides *Sphaerotilus* a more competitive advantage than other organisms.

The other factors ascribed to sludge bulking, which include stale sewage, septic return sludge and high temperature at low flow rate of waste, can be related to the low oxygen tension in the mixed liquor and high concentration of H_2S in the received sewage.

Sludge volume indexes (SVI) ranging between 100 and 2,000 ml/g have been observed as a result of filamentous bulking (Pipes, 1967b; Kraus, 1949). However, bulked sludge cuased by an excessive growth of fungi never had an SVI of over 300 ml/g (Pipes, 1967a). The activated sludge process can be successfully operated in a bulked condition provided the plant has

secondary clarifiers and sludge recirculation pumps of adequate capacity (Pipes, 1967a; Bloodgood, 1947). When such a system is properly operated, the effluent contains less BOD and suspended solids than is usually obtained with normal sludge (Haseltine, 1932; Heukelekian, 1941; Keefer, 1963). Bhatla (1967) reporting on experience with a 10 MGD plant treating kraft mill waste indicated that when the plant was operated in a bulking condition, BOD and suspended solids removals were 5 to 10 percent greater than when operated in a nonbulking state.

Pfeffer (1967) studies the role of iron and other metals in the growth of the fungi *Fusarium* and *Geotrichum*. These were pure and mixed culture studies. In the continuous flow mixed culture studies, iron and manganese addition to glucose substrate inhibited the growth of these filamentous fungi and produced a sludge with good settling characteristics.

The role of iron in the activated sludge was further investigated by Carter and McKinney (1973). These researchers indicated that supplying adequate iron in the activated sludge system reduced sludge bulking problems and produced a higher rate of metabolism.

Since the oxygenation of ferrous iron occurs rapidly under neutral pH and aerobic condition, most of the iron added in the activated sludge system would be oxidized to ferric form and precipitated as ferric hydroxide. The mechanism for reducing bulking problems by adding iron in the activated sludge systems may be partly due to the precipitation of ferric hydroxide on the filamentous organisms. The precipitated iron on the microbial surfaces will tend to block the transfer of the organic nutrients across the microbial interface and inhibit the growth of these organisms. Actually, Carter and McKinney (1973) noticed this mechanism in their research. These researchers

found that increasing the addition of iron from 2 mg/l up to 10 mg/l increased the rate of metabolism in the activated sludge system while 20 mg/l of iron had adverse effect. Low concentration of iron stimulates the growth of normal organisms and inhibits the growth of filamentous organisms while high concentration of iron inhibits both.

2. *Sphaerotilus*

Sphaerotilus was first described by Kützing (1833) as an algae similar in appearance to *Melosisa* or *Fragilaia*. The taxonomy of this organism is still confused. The generally accepted description of this organism is that from Phaup (1968). The genus *Sphaerotilus* is composed of Gram-negative, nonsporulating rods enclosed in a sheath, which may or may not show false branching. The sheaths may be encrusted with iron or manganese salts, which impart a brownish or blackish color to them. Members of the genus reduce nitrates to nitrites but not to ammonia; gelatin is liquefied slowly; many compounds may serve as a carbon-energy source; and growth occurs with either organic and inorganic nitrogen.

Sheath formation in the presence of ferric iron is the general characteristic of this organism. Romano and Peloquin (1963) determined the composition of the sheath of *Sphaerotilus* and found it similar to the cell walls of many bacteria with the exception that muramic acid was not detected. They described the sheath material as a protein-polysaccharide-lipid complex distinct from cell wall and slime layer materials. Romano and Geason (1964) observed the pattern of sheath synthesis by means fo fluorescent microscopy and concluded that sheath synthesis occurs by linear extension of existing sheath. The composition of the sheath may vary with nutrition. Gaudy and

Wolfe (1962) indicated that as the available organic nitrogen was increased, the production of capsular material was stimulated while the sheath formation was inhibited. Skerman (1959) suggested that capsular material might be somehow incorporated directly into the sheath. Such an occurrence seems possible due to the relationship of capsule and cell wall and the similarities of their chemical composition. Ferric and manganic salts may be deposited on the outer surface (Pringsheim, 1949; Mulder and van Veen, 1963). The iron deposition on to the sheaths of heat-killed cultures indicated that at least part of such deposition was physical rather than biological (Phaup, 1968).

Cell size varies considerably. The dimensions most often reported are 1-2.5 μm in width and 3-8 μm long. In "young" cultures, cells are usually larger and cross-walls difficult to see (Stokes, 1954). The cytoplasm of young cells is usually clear, becoming granular in older cultures. Phaup (1968) noted the disappearance of these granules in cultures on agar stored 30 days or longer at 4°C, and in cells subjected to anaerobiosis for several days.

Rouf and Stokes (1962), using the method of Williamson and Wilkinson (1958), isolated the granules of *Sphaerotilus* and identified them as poly-β-hydroxybutyrate, a reserve bacterial food. Stokes and Powers (1967b) stated that *S. discophorus* synthesized large amounts of this polymer when grown on glucose-containing media. They obtained a considerable increase in the rate of endogenous oxygen consumption in these cells through the addition of Mn^{++} and even greater increase after addition of Mg^{++}. These ions undoubtedly stimulate the oxidation of poly-β-hydroxybutrate, which probably occurs via hydrolysis to β-hydroxybutyrate, oxidation of the

latter to acetoacetate, and subsequent oxidation of acetoacetate with Coenzyme A through the tricarboxylic acid cycle.

Skerman, Dementjeva and Carey (1958) described granules occurring along the longitudinal axis of the cell which were involved in the intracellular deposition of sulfur when *Sphaerotilus* was exposed to H_2S. Phaup (1968) found that cells containing large amounts of sulfur were nonviable and proposed that this phenomenon might be a mechanism for removal of toxic H_2S from their environment rather than an energy-yielding bio-oxidation process. This assumption was substantiated by the observation of Waitz and Lackey (1959); *Sphaerotilus* deposited sulfur in the presence of SO_2. It would seem, then, that there are at least two types of intracellular granules; e.g., the scattered granules of poly-β-hydroxybutyrate, and the longitudinally arranged sulfur granules.

The nutrition of *Sphaerotilus* has been investigated extensively by Stokes (1954), Mulder and van Veen (1963), Razumov (1961), and Lackey and Wattie (1940). The mineral salt requirements were reported to be similar to other bacteria. Stokes (1954) used a basal solution with the following components: $MgSO_4 \cdot 7H_2O$, 0.02%; $CaCl_2$, 0.005%; $FeCl_3 \cdot 6H_2O$, 0.001%; phosphate buffer 0.01 M. Lackey and Wattie (1940) employed Na_2HPO, 0.005%; NaCl, 0.0015%; KCl, 0.0007%; $CaCl_2$, 0.0007%; and $MgSO_4$, 0.0005%. Razumov (1961) employed $CaCl_2$, 0.002%; $MgSO_4$, 0.002%; K_2HPO_4, 0.002% and $FeCl_3$, 1 drop of 1% solution.

Many organic compounds were reported to be used as a sole source of carbon by *Sphaerotilus*. They were: glucose, galactose, maltose and sucrose among the sugars (Lackey and Wattie, 1940; Razumov, 1961; Stokes, 1954); succinate, fumarate, lactate, pyruvate and acetate among the acids (Stokes, 1954); ethanol, butanol, and glycerol among the alcohols (Stokes, 1954).

However, this organism seems to utilize glucose more readily than other organic compounds. Oxidation of sugars, amino acids and compounds of the tricarboxylic acid cycle was repressed by addition of glucose (Stokes and Powers, 1967a).

Preference for organic nitrogen rather than inorganic nitrogen as nitrogen source was reported by most investigators (Stokes, 1954; Lackey and Wattie, 1940; Mulder and van Veen, 1963; Razumov, 1961). Phaup (1968) reported that amino acids providing suitable nitrogen for growth were: asparagine, alanine, arginine, glutamic acid, methionine, proline, and probably leucine; not suitable, or toxic, were serine, cystine, histidine, lysine, ornithine, threonine, tryptophane, tyrosine, and valine.

The desirable pH range for this organism was reported from 6.0 to 9.0 (Stokes, 1954; Lackey and Wattie, 1940). Growth of *Sphaerotilus* has been reported to occur at temperatures from 5 to 40°C with the optimal growth at 30°C (Stokes, 1954). Although this organism would not grow under anaerobic conditions, it could grow in extremely low oxygen tensions (Stokes, 1954).

Waitz and Lackey (1959) observed that the growth of *Sphaerotilus* was inhibited with a concentration of 25 mg/l of ferric chloride. Since *Sphaerotilus* is often called an iron-bacterium due to its ability to deposit iron in the form of iron oxide along its sheath, the role of iron in the inhibition of this organism is an interesting problem.

III. CHEMISTRY OF IRON

1. Redox Equilibria

Thermodynamic considerations are useful for obtaining a general understanding of the potential reactions of iron. The following equation is a general expression for the thermodynamic reactions in aqueous solutions.

$$p\varepsilon = p\varepsilon^\circ - \frac{1}{n} \log Q \qquad (1)$$

where $p\varepsilon$ = relative electron activity ($p\varepsilon$ = -log e). Large positive values of $p\varepsilon$ (low electron activity) represent strongly oxidizing conditions while small or negative values (high electron activity) correspond to strongly reducing conditions. $p\varepsilon^\circ$ = relative electron activity when all species other than the electrons are at unit activity. Q = reaction quotient.

$p\varepsilon$ is a measure of the free energy involved in the transfer of electrons:

$$p\varepsilon = - \frac{\Delta G}{n2 \cdot 3RT} \qquad (2)$$

When the free energy change ΔG is in kilocalories at 25°C, the relation becomes

$$p\varepsilon = - \frac{0.7342 \Delta G}{n} \qquad (3)$$

The following relations are also important in calculating $p\varepsilon$ values from free energy and electrode potential data,

$$p\varepsilon^\circ = - \frac{0.7342 \Delta G^\circ}{n} \qquad (4)$$

$$p\varepsilon^\circ = \frac{1}{n} \log K \qquad (5)$$

$$p\varepsilon^\circ = 16.93\ E^\circ \qquad (6)$$

where n = the number of electrons transferred in the reaction

ΔG° = standard free energy change at 25°C, in kilocalories per mole

K = equilibrium constant

E° = standard reduction potential at 25°C, in volts per mole of electron transferred

By employing the equations 1 through 6 and the values of free energy given in Table 1 and the electrode potential in Table 2, the activity ratio diagrams and $p\varepsilon$ - pH diagrams for iron can be established. Activity ratio diagrams for iron (Figure 1) give a rapid survey on the stability relations at pH 7.0 and for $[HCO_2^-] = 10^{-3}$ M and $[SO_4^{2-}] = 10^{-3}$ M. Because of the uncertainty of free energy data, especially for the various iron oxides, the positions of the lines are not exact. There is considerable uncertainty about the $p\varepsilon$ values at which equilibrium between Fe_2O_3 and Fe_3O_4 occurs. The diagrams, furthermore, suggest that iron sulfides start to be formed as one passes to $p\varepsilon$ values lower than -2 to -3. Even if sulfides are being formed, the sulfate concentration in many natural waters does not vary appreciably; hence, the assumption of a constant sulfate concentration is justified. But if $p\varepsilon$ values drop further (below $p\varepsilon = -3$ at pH = 7), $[SO_4^{2-}]$ does not remain constant but decreases.

A more extensive summary of limiting stability relations for iron in the light of presently available thermodynamic information is given by the $p\varepsilon$ - pH diagram shown in Figure 2 for a set of specified conditions. At pH above -10, metallic iron and its common alloys tend to be oxidized to ferrous and ferric states. Under aerobic conditions, and neutral pH, $Fe(OH)_3$ is the predominant form.

Table 1. Iron Species in Aquatic Environment and their Free Energy of Formation (Stumm and Morgan, 1970)

Formula	Description	State	$\bar{G}_f^\circ$ at 25°C Kcal mole^{-1}
Fe(II)			
Fe^{2+}		aq	-20.3
$FeSiO_3$		s	-257
Fe_2SiO_4		s	-319.8
α-FeS		s	-23.32
FeS	pyrite	s	-39.9
$Fe(OH)_2$		s	-115.57
$FeCO_3$	siderite	s	-160.5
Fe(III)			
Fe^{3+}		aq	-2.53
$Fe(OH)_3$		s	-117.1
(amorph)-FeOOH		s	-111.1
α-FeOOH	geothite	s	-113.7
α-Fe_2O_3	haematite	s	-177.1
$FePO_4 2H_2O$	strengite	s	-279
Fe(II), Fe(III)			
Fe_3O_4	magnetite	s	-242.4

Table 2. Equilibrium Constants for Redox Reaction of Iron (Stumm and Morgan, 1970)

Half-reaction	$p\varepsilon^\circ$	Electrode potential volts 25°C
$1/2\ Fe^{2+} + e = Fe(s)$	-7.5	-0.44
$Fe^{3+} + e = Fe^{2+}$	+13.2	-0.77
$Fe(OH)_3(s) + 3H^+ + e = Fe^{2+} + 3H_2O$	+18.8	+1.06

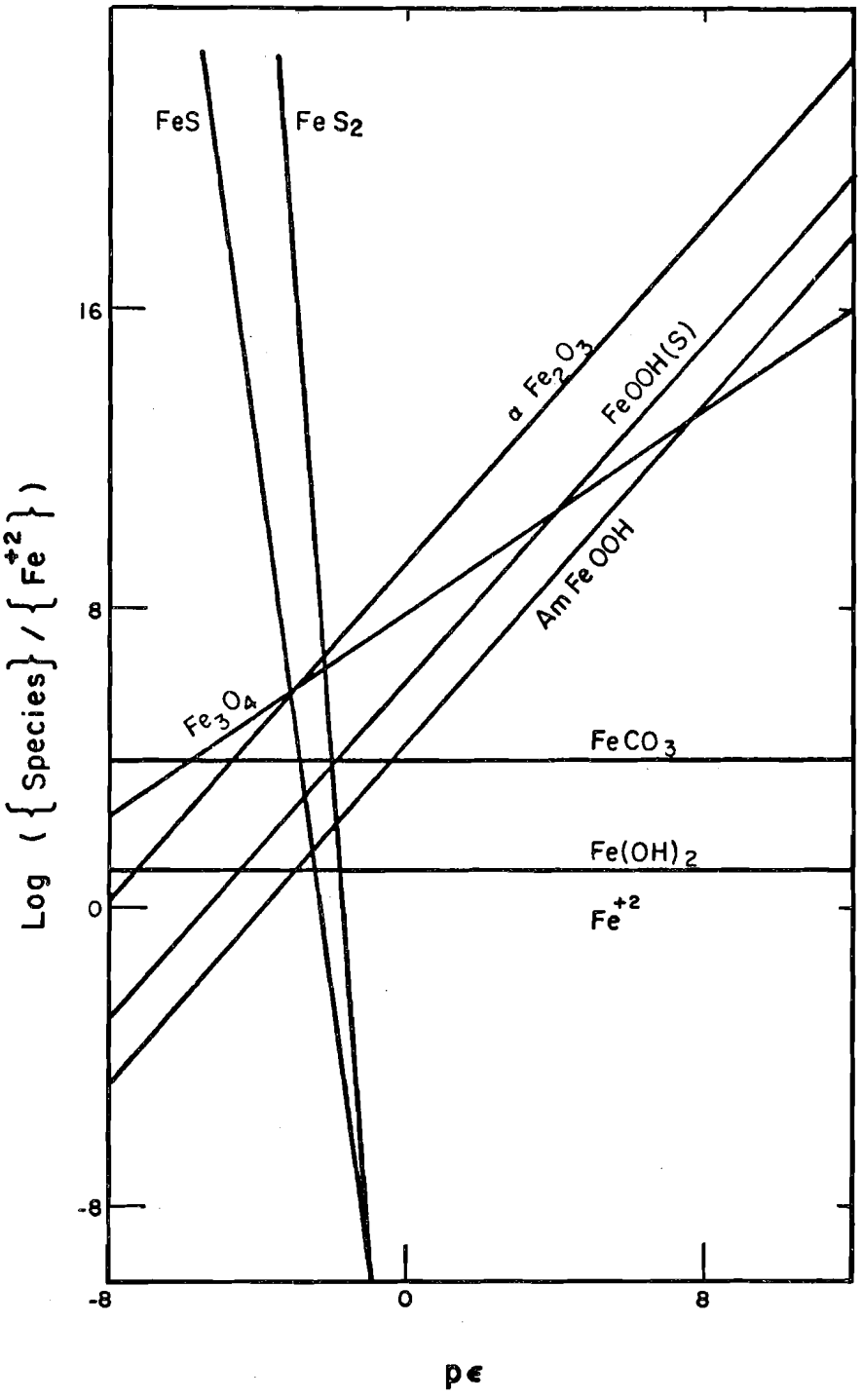


Figure 1. Activity Ratio Diagram for Iron, pH = 7.0, $C_T = 10^{-3}$ M and $[SO_4^{2-}] = 10^{-3}$ M
(Stumm and Morgan, 1970)

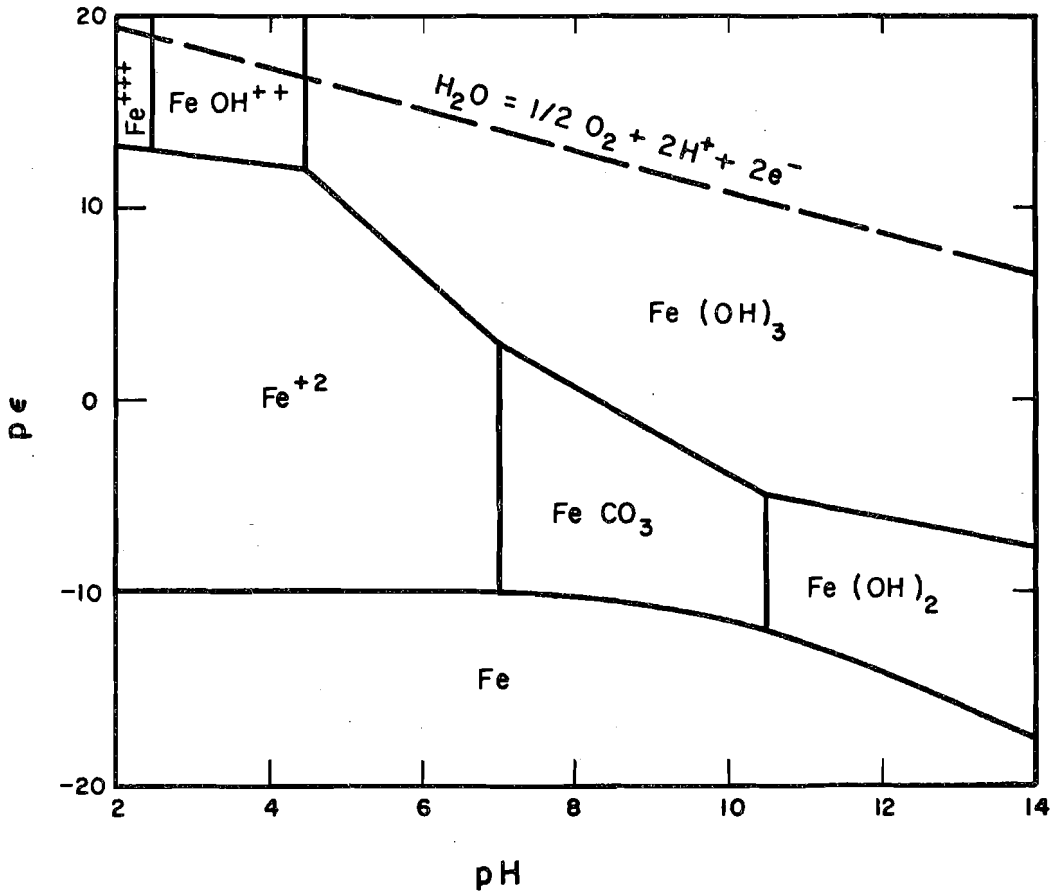


Figure 2. pε-pH Diagram for Iron, $C_T = 2 \times 10^{-3}$ M, Activity of Soluble Metal Ion Species, 10^{-3} M (Stumm and Morgan, 1970)

2. Kinetics of Redox Reactions

The rate of oxygenation of Fe(II) in solutions of pH $\geq$ 5.5 was found to be first order with respect to the concentrations of both Fe(II) and O_2 and second order with respect to the OH ion. Thus a 100-fold increase in the rate of reaction occurs for a unit increase in pH. Catalysts (especially Cu^{2+}, Co^{2+}) in trace quantities, as well as anions which form complexes with Fe(II) (e.g., HPO_4^{2-}, citric acid), increase the reaction rate significantly. The oxygenation kinetics obey the following rate expression (Stumm and Lee, 1961).

$$\frac{-d[Fe(II)]}{dt} = k[Fe(II)][OH^-]^2 P_{O_2} \qquad (7)$$

Frequently it is more convenient to use the rate expression in the form

$$\frac{-d \ln [Fe(II)]}{dt} = \frac{k_H [O_2(aq)]}{[H^+]^2} \qquad (8)$$

where k_H at 20°C = 3×10^{-12} min^{-1} mole $liter^{-1}$. For a given pH, the rate increases about tenfold for a 15°C temperature increase. For constant $[H^+]$, an activation energy of 23 kcal $mole^{-1}$ can be calculated.

In the low pH range (pH < 4), the rate of oxidation is independent of pH. The rate of oxygenation of ferrous iron over the entire pH range is shown in Figure 3. The oxidation reaction is catalyzed by interfaces and by light. In the presence of light the reaction is approximately 2-3 times as fast as in its absence. Substantial surface area concentrations ($S > 100$ m^2 $liter^{-1}$) are necessary to enhance the oxidation reaction markedly.

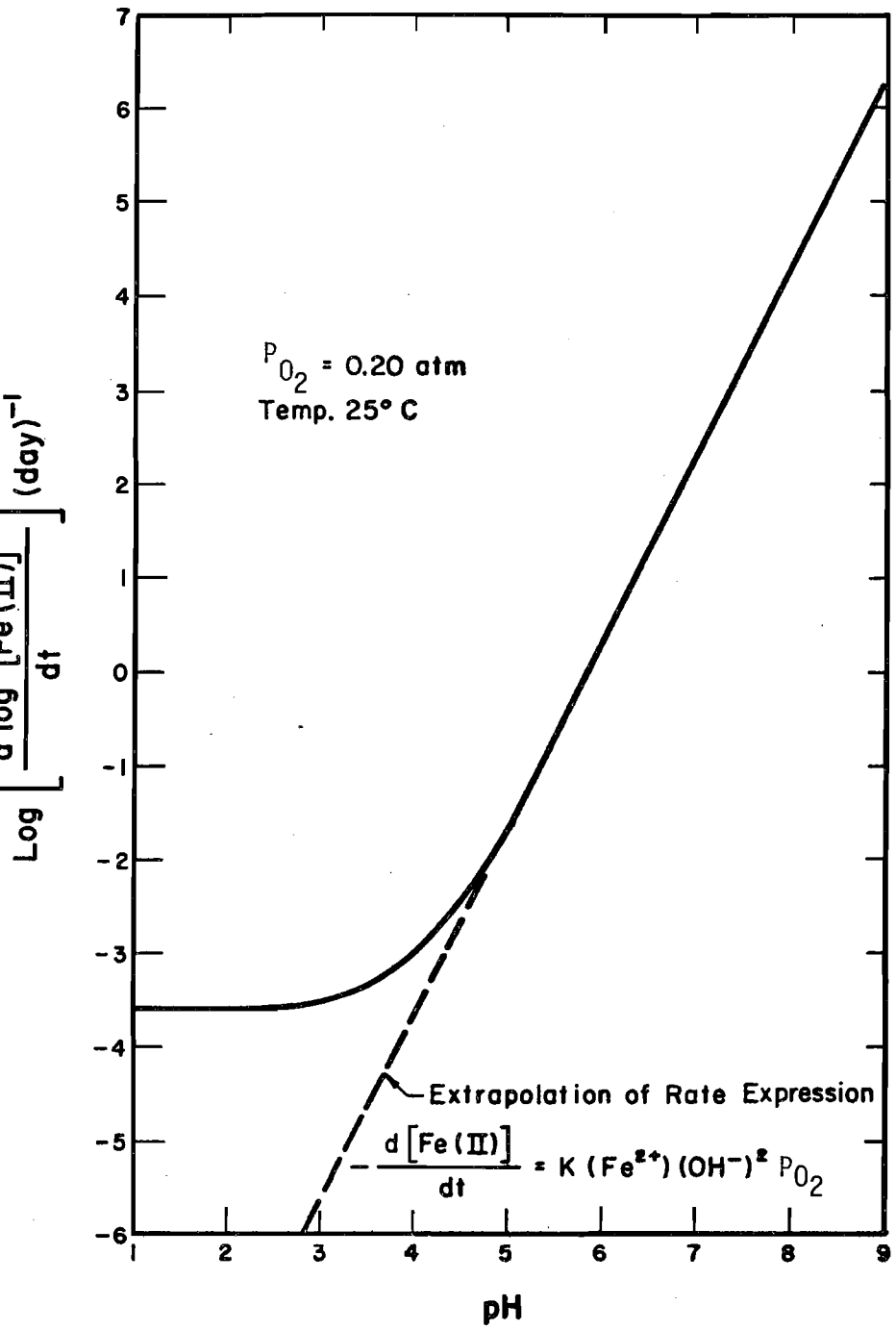
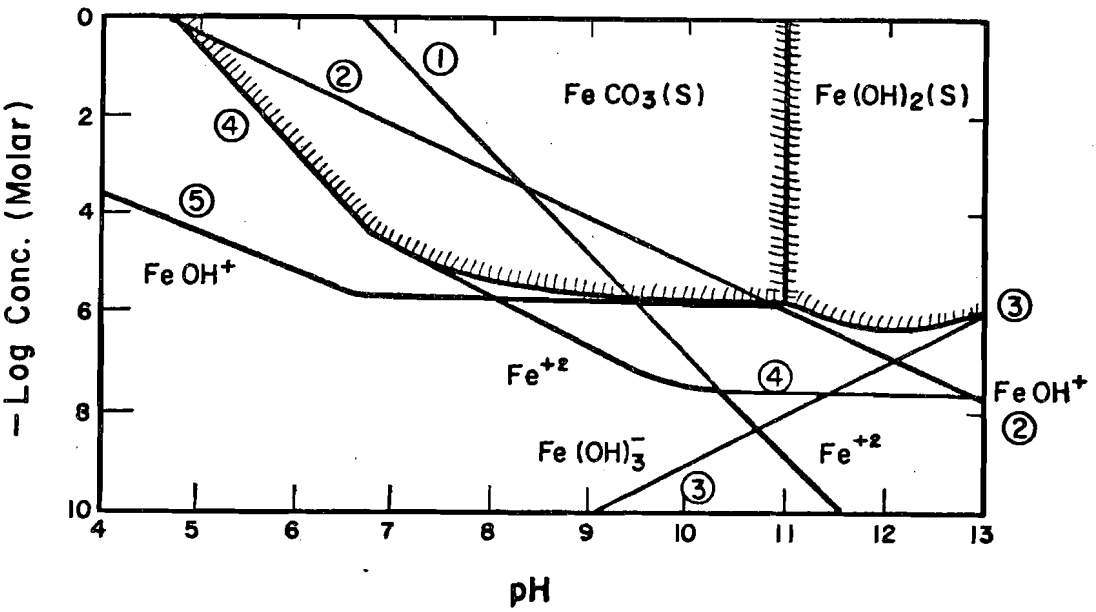


Figure 3. Oxygenation Rate of Ferrous Iron as a Function of pH (Stumm and Morgan, 1970)

3. Solubility and Complex Formation

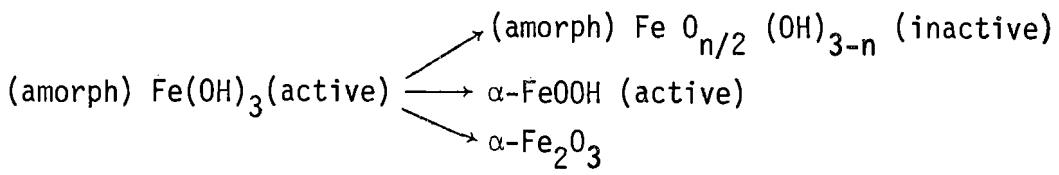
The solubility of iron in the water depends on pH of the water, oxidation-reduction potential of the environment ($p\varepsilon$), species of anions, organic compounds and bacterial activities. For systems closed to the atmosphere, a solubility diagram can conveniently illustrate the conditions under which a particular solid phase predominates. Figure 4 gives a solubility diagram for Fe(II) considering $FeCO_3(s)$ as possible solid phases. At pH below 11, Fe(II) precipitates as $FeCO_3$, while $Fe(OH)_2$ limits soluble iron above pH 11. In the presence of H_2S, Fe(II) forms insoluble FeS and FeS_2 (see Figure 1).

As the oxidation potential of the environment increases, ferrous iron will be oxidized to ferric forms and precipitate as ferric hydroxide. The precipitates often occur in amorphous and several crystalline modifications. An "active" form of the precipitates, that is, a very fine crystalline precipitate with disordered lattice, is generally formed incipiently from strongly oversaturated solutions. Initially formed amorphous precipitates or active forms of unstable crystalline modifications may undergo two kinds of changes during aging. Either the active form of the unstable modification becomes inactive or a more stable modification is formed. The deactivation may be accompanied by condensation. When several of the processes take place together, nonhomogeneous solids are formed upon aging. In dissolution experiments with such nonhomogeneous solids, the more active forms are dissolved more readily. Measurements of the solubility or "active" forms give solubility products that are higher than those of the inactive forms. Feitknecht and Schindler give the following example (Stumm and Morgan, 1970):



1. $Fe(OH)_2\ (S) \rightleftarrows Fe^{+2} + 2\ OH^-$ $\quad K_{s0} = 2 \times 10^{-15}$
2. $Fe(OH)_3\ (S) \rightleftarrows Fe(OH)^+ + OH^-$ $\quad K_{s1} = 4 \times 10^{-10}$
3. $Fe(OH)_2\ (S) + OH^- \rightleftarrows Fe(OH_3)^-$ $\quad K_{s3} = 8.3 \times 10^{-6}$
4. $FeCO_3\ (S) \rightleftarrows Fe^{+2} + CO_3^{-2}$ $\quad K_{s0} = 2.1 \times 10^{-11}$
5. $FeCO_3\ (S) + OH^- \rightleftarrows FeOH^+ + CO_3^{-2}$ $\quad K_{s1} = 0.1 \times 10^{-5}$

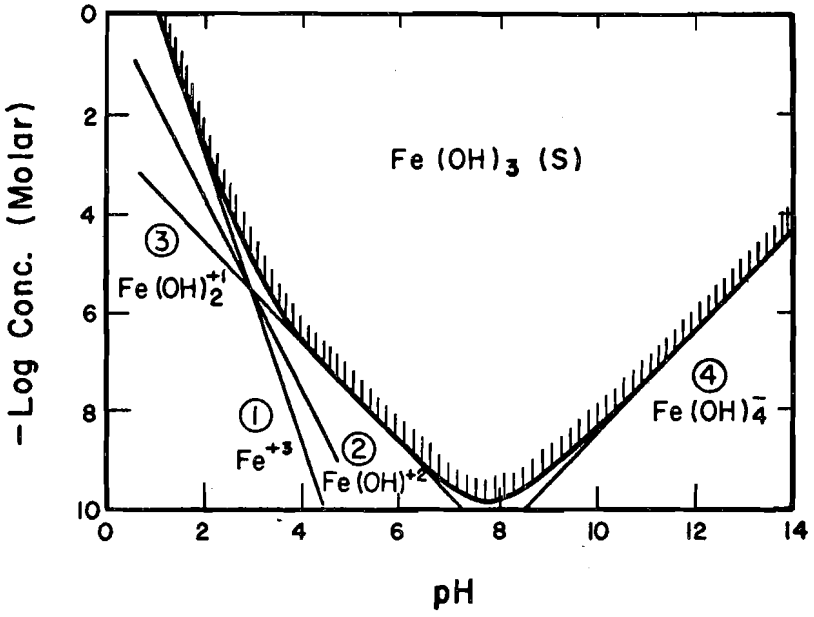
Figure 4. Solubility of $Fe(OH)_2(S)$ and $FeCO_3(S)$ (siderite) 25°C; I = 0, $C_T = 10^{-3}$ M (Stumm and Morgan, 1970)



In determining solubility equilibrium constants, many investigators have been motivated by a need to gain information that was pertinent primarily for relatively short-term conditions (minutes to hours) typically encountered in the laboratory. In operations of analytical chemistry, for example, precipitates are frequently formed from strongly oversaturated solutions; the conditions of precipitation of the incipient active compound rather than the dissolution of the aged inactive solid are often of primary interest. Most solubility products measured in such cases refer to the most active component. On the other hand, in dealing with heterogeneous equilibria of natural water systems, the more stable and inactive solids are frequently more pertinent.

Among the oxides and hydroxides of iron, one needs to consider primarily $Fe(OH)_2$, Fe_3O_4 (magnetite), amorphous FeOOH and α-FeOOH (geoethite). What is usually called "ferric hydroxide" is more likely a poorly crystallized FeOOH. Data for the solubility and free-energy of formation of $Fe(OH)_3$(s), probably based on results obtained with active FeOOH preparations, are nevertheless of operational value. A solubility diagram for Fe(III) considering ferric hydroxide as possible solid phase is given in Figure 5.

Complex formation of Fe(III) with ortho-phosphate and many organic bases is well established. In order to evaluate the coordinating tendency of Fe(III), the relative affinities of Fe^{3+} for OH^- ions and other ligands need to be compared. Hydroxide ions often have a stronger affinity for



		pK
1.	$Fe(OH)_3\ (S) \rightleftarrows Fe^{+3} + 3\ OH^-$	38.7
2.	$Fe(OH)_3\ (S) \rightleftarrows FeOH^{+2} + 2\ OH^-$	27.5
3.	$Fe(OH)_3\ (S) \rightleftarrows Fe(OH)_2 + OH^-$	16.6
4.	$Fe(OH)_3\ (S) + OH^- \rightleftarrows Fe(OH)_4^-$	4.5

Figure 5. Solubility of $Fe(OH)_3$
25°C; I = 3 M $NaClO_4$
(Stumm and Morgan, 1970)

Fe^{3+} than do organic or inorganic bases. The extent of complex formation is thus pH dependent, and, within the pH range of natural waters, soluble or insoluble mixed Fe(III) complexes that may contain OH^- as well as other ligands can be formed. For example, Fe(III) interacts chemically with orthophosphate to form soluble phosphate-iron (III) complexes [e.g., $FeHPO_4^+$, $Fe_x\ (H_nPO_4)_y^{3x-(3-n)y}$]. Under slightly acid conditions pure $FePO_4(s)$ will be precipitated, whereas in the neutral and slightly alkaline pH range the precipitate is probably a ferric compound containing both PO_4^{3-} and OH^- in variable proportions, depending upon the pH. The chemical interaction of Fe(III) with many organic bases are similar to that with orthophosphate. Some stability constants of iron complexes are given in Table 3 and Table 4.

4. Interaction with Organic Substances

The reactions of iron with organic compounds can be divided into two groups, oxidation reaction and chelation reaction, based on whether the organic compound is oxidized during the reaction or not. Qualitative and quantitative experiments have confirmed that S (-II) compounds (HS^-, cysteine) as well as a variety of organic substances, especially such compounds as phenols, polyphenols, gallic acid, tannic acid, can reduce ferric iron reasonably fast in synthetic solutions (minutes to hours) (Theis and Singer, 1974). If conditions (pH, concentrations) are such that the rate of Fe(II) oxygenation is slow in comparison to the Fe(III) reduction by the organic material, a relatively high steady-state concentration of Fe(II) can be maintained in the system as long as the organic material is not fully oxidized. This observation may be

Table 3. Stability Constants of Iron Complexes (Sillen and Martell, 1964)

Reaction	Symbol for Equilibrium Constant	log K(25°C)	I
(am) $Fe(OH)_3(s) = Fe^{3+} + 3OH^-$	K_{so}	-38.7	3M $NaClO_4$
(am) $Fe(OH)_3(s) = FeOH^{2+} + 2OH^-$	K_{s1}	-27.5	3M $NaClO_4$
(am) $Fe(OH)_3(s) = Fe(OH)_2^+ + OH^-$	K_{s2}	-16.6	3M $NaClO_4$
(am) $Fe(OH)_3(s) + OH^- = Fe(OH)_4^-$	K_{s4}	-4.5	3M $NaClO_4$
2(am) $Fe(OH)_3(s) = Fe_2(OH)_4^{4+} + 4OH^-$	K_{s22}	-51.9	3M $NaClO_4$
(am) $FeOOH(s) + 3H^+ = Fe^{3+} + 2H_2O$	K_{so}	3.55	3M $NaClO_4$
α-$FeOOH(s) + 3H^+ = Fe^{3+} + 2H_2O$	K_{so}	1.6	3M $NaClO_4$
$Fe(OH)_2$(active) $= Fe^{2+} + 2OH^-$	K_{so}	-14.0	0
$Fe(OH)_2$(inactive) $= Fe^{2+} + 2OH^-$	K_{so}	-14.5	0
$Fe(OH)_2$(inactive) $+ OH^- = Fe(OH)_3^-$	S_{s3}	-5.5	0
$FeCO_3$(siderit) $= Fe^{2+} + CO_3^{2-}$	K_{so}	-10.4	0
$FeCO_3(s) + OH^- = Fe(OH)^+ + CO_3^{2-}$	K_{s1}	10^{-6}	0
$FeS = Fe^{2+} + S^{2-}$	K_{so}	$10^{-17.3}$	
$Fe_3(PO_4)_2 = 3Fe^{2+} + 2PO_4^{3-}$	K_{so}	$10^{-29.9}$	
$FePO_4 = Fe^{3+} + PO_4^{3-}$	K_{so}	10^{-23}	
$FeHPO_4^+ = Fe^{3+} + HPO_4^{2-}$	K	$10^{-8.3}$	
$Fe(HP_2O_7)_2^{3-} = Fe^{3+} + 2HP_2O_7^{3-}$	K	10^{-22}	

Table 4. Stability Constants of Some Iron-Organic Complexes (Sillen and Martell, 1964)

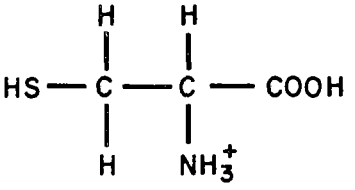
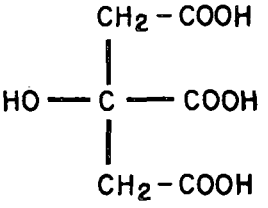
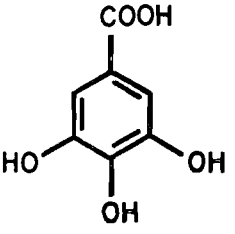
Structure		Symbol
	Cysteine	H_3L^+
	Citrate	H_4L
	Gallic Acid	HL

Table 4 (Continued)

Stability Constants

Cysteine

Reaction	Description	log K	I
$H_3L^+ = H_2L + H^+$	-COOH	-1.71	0
$H_2L = HL^- + H^+$	sulfur group	-8.60	0
$HL^- = L^{2-} + H^+$	amino group	-10.51	0
$FeL_3^{3-} = Fe^{3+} + 3L^{2-}$		-32.10	0
$FeOHL_2^{2-} = Fe^{3+} + 2L^{2-} + OH^-$		-33.30	0

Citrate

Reaction	Description	log K	I
$H_4L = H_3L^- + H^+$	-COOH	-3.02	0.1 M $NaNO_3$
$H_3L^- = H_2L^{2-} + H^+$	-COOH	-4.50	0.1 M $NaNO_3$
$H_2L^{2-} = HL^{3-} + H^+$	-COOH	-5.80	0.1 M $NaNO_3$
$HL^{3-} = L^{4-} + H^+$	-OH	-16.0	0.1 M $NaNO_3$
$FeH_2L^+ = H_2L^{2-} + Fe^{3+}$		-6.31	0.1 M $NaNO_3$
$FeHL = HL^{3-} + Fe^{3+}$		-11.7	0.1 M $NaNO_3$
$FeL^- = L^{4-} + Fe^{3+}$		-25	0.1 M $NaNO_3$

Gallic Acid*

Reaction	Description	log K	I
$HL = L^- + H^+$	-COOH	-4.41	0

* Source: Hand Book of Chemistry and Physics. The Chemical Rubber Co., 18901 Cranwood Parkway, Cleveland, Ohio 44128.

illustrated by the following types of reaction sequence:

$$\text{Fe(III)} + \text{org} \rightarrow \text{Fe(III)-org complex}$$

$$\text{Fe(III)-org} \rightarrow \text{Fe(II)} + \text{oxidized org}$$

$$\text{Fe(II)} + 1/4\ O_2 \rightarrow \text{Fe(III)}$$

In these cases the ferric-ferrous system acts merely as a catalyst for the oxidation of organic material by oxygen.

Certain organic compounds such as citric acid, oxalic acid, ethylenediaminetetraacetate (EDTA), 1,2-diaminocyclonexanetetraacetate, and nocordamine may chelate ferric iron and prevent the iron from precipitating. The organic complexes of iron, like the inorganic complexes, dissociates in water and reach equilibrium with their counterparts. Because of the importance of iron complexes with cysteine, citrate and gallic acid in this research, the stability constants of these complexes are given in Table 4. The so-called peptizing reaction of Fe(III) dispersions with organic material is probably the result of the chelating of organic material on the hydrolyzed ferric iron.

5. Surface Chemistry of Ferric Hydroxide

The precipitates of iron hydroxides can interact with organic and inorganic anions as well as the soluble species of iron. The incorporation of coordinating anions into basic precipitates strongly affects the colloid chemical properties of the dispersed phase. An enhancement of the colloid stability of these suspensions frequently results. It is usually difficult to distinguish operationally by conventional means (membrane filtration, centrifugation, dialysis) between homogeneous phase soluble Fe(III) complexes and peptized Fe(III) dispersions. In natural waters high

concentrations of organic material are frequently associated with high concentrations of operationally "soluble" iron (III). Most of the so-called natural color of water is probably ascribable to highly stabilized colloidal dispersions where the intensive yellow staining is caused partially by complex formation with hydrolyzed ferric iron.

The zero point of charge (ZPC) is convenient reference for predicting the charge dependent behavior of the suspensions of oxide minerals. The ZPC is the pH at which the solid surface charge from all sources is zero. Below this pH, the oxides carry a positive charge, while they carry a negative charge above this pH. The ZPC of a complex oxide is approximately the weighted average of the ZPC of its compounds. Predictable shifts in ZPC occur in response to specific adsorption and to changes in cation coordination, crystallinity, hydration state, cleavage habit, surface composition, and structural charge or ion exchange capacity. α-Fe_2O_3 prepared by hydrolysis and not subsequently dried at high temperature has the ZPC of 8.5 and apparently retains a coating of α-FeOOH (Parks, 1965; 1967).

Hydrous Fe oxides suspended in waters are frequently present as amorphous or microcrystalline form, characterized by high specific surface area. Areas up to 300 m^2g^{-1} have been reported for α- and γ-FeOOH. These hydrous oxides, especially at pH values higher than pH_{zpc}, are capable of interacting with cations. Sorption of metal ions to these oxides may properly be interpreted as surface complex formation or as ion exchange since hydrogen ions or other cations are released as metal ions become adsorbed. The adsorption has been found to be strongly dependent on pH. While adsorption with Group I and II cations take place predominantly in

the diffuse part of the electrical double layer, the transition and heavy metal ions become specifically attached to the surface. Representative results of studies on the equilibrium of the pH-dependent sorption of Mn(II) on $Fe(OH)_3$ suspensions are presented in Figure 6.

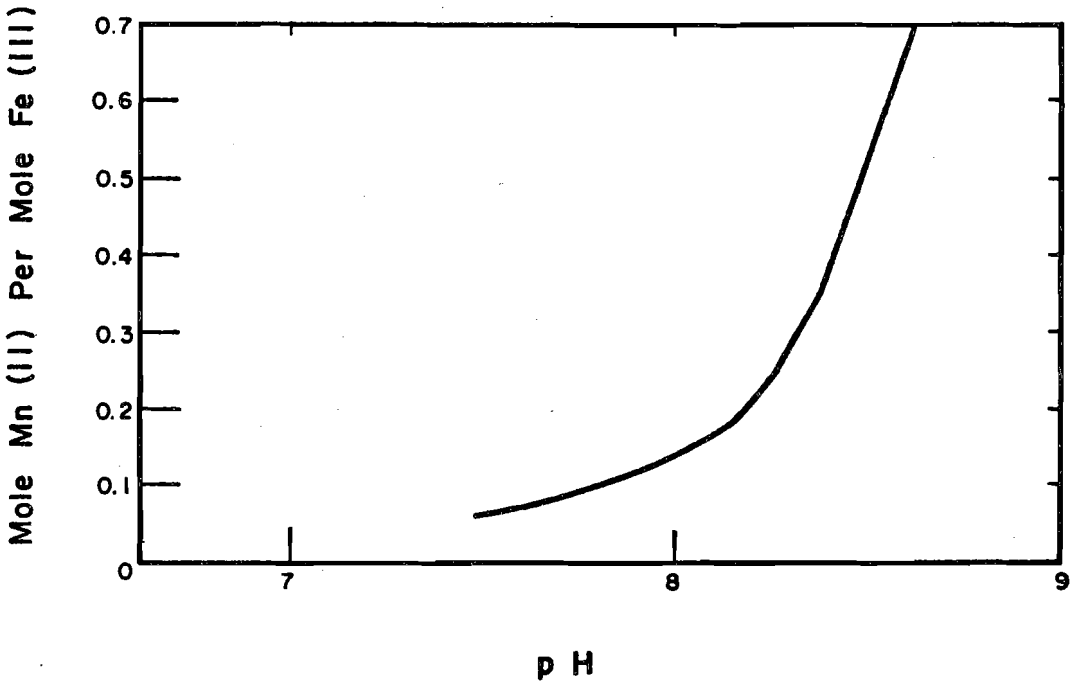


Figure 6. Sorption Characteristics of Fe(III) Oxides, 25°C (Stumm and Morgan, 1970)

IV. EXPERIMENTAL PROCEDURE

1. Culture Media

The solid medium used for the isolation of the *Sphaerotilus* was prepared according to Stokes (1954). The synthetic sewage (S-medium) formulated by Lackey and Wattie (1940) was employed in all growth studies. This medium was modified somewhat for different studies. The compositions of these media are shown in Table 5.

Table 5. A Comparison of Stokes' Medium with Lackey and Wattie's Medium

Compounds	Stokes' medium		Lackey and Wattie's medium (iron enriched)
	%	mg/l	mg/l
glucose	0.1	1,000	500
peptone	0.1	1,000	100
$MgSO_4$	0.01	100	5.0
$CaCl_2$	0.005	50	7.0
Na_2HPO_4	-	-	50
NaCl	-	-	15
K Cl	-	-	7.0
water	tap water		distill water
agar	1.25		-
$FeCl_3$	0.0006	6.0	6.0

2. Isolation of *Sphaerotilus*

Sludge bulking was induced in a laboratory activated sludge unit receiving a substrate consisting primarily of dextrose and peptone dissolved in tap water. When the sludge volume index exceeded 500 and the microbial flora appeared to contain a substantial population of *Sphaerotilus* the effluent from the activated sludge unit was streaked on the Stokes medium and incubated at 37°C. Pure cultures were obtained by restreaking fresh plates with isolated colonies that appeared to be *Sphaerotilus*. The isolated colonies were confirmed to be *Sphaerotilus* according to the description presented by Pelezar and Reid (1972). The procedures of Farquhar and Boyle (1971a) was used to determine the following organism characteristics:

1. Sulfur deposits
2. Presence of a sheath
3. Iron oxidation
4. Lipid deposits

Pure cultures were maintianed on solid medium at 5°C and transferred weekly. In order to verify the identification of the isolated organism, a species of *Sphaerotilus** was obtained from the American Type Culture Collection for comparative purposes.

The inoculum of *Sphaerotilus* used in the pure culture studies was prepared prior to each test run according to the following procedure:

1. Distribute S-medium in 50 ml quantities in a pair of 250 ml flasks and sterilize.
2. Inoculate one of the flasks with *Sphaerotilus* from a solid medium culture.

**Sphaerotilus* natans Kutzing 15291, The American Type Culture Collection 12301 Parklawn Drive, Rickville, Maryland.

3. Shake for 9 hours at room temperature.
4. Examine culture microscopically to verify pure culture.
5. Transfer 3 ml of this culture to the other flask and shake for 9 hours.
6. This second culture is the inoculum for the pure culture studies.

This procedure was designed to minimize the experimental error of inoculation by providing an inoculum with a uniform concentration of acclimated cells.

3. Respiration Rates

The standard Warburg manometric technique was used to determine the respiration rate (growth rate) of the microorganisms under different test conditions. The respirometer employed in this study permitted precise temperature control from 0°C to 60°C. The unit had 14 flasks and manometers which permitted testing a wide range for a given parameter at one time.

4. Iron-Organic Interaction

When iron is added to the culture medium, several reactions are possible. Some of the iron may precipitate as ferric hydroxide or other insoluble iron salts. Certain compounds in the culture medium may chelate the iron, keeping it in solution as an organic chelate.

According to Theis and Singer (1974), the procedure by which the iron is added has a determinant effect on the formation of the iron-organic complex. When ferric iron was added to tannic acid (10^{-4} M) in the form of crystals of ferric perchlorate [$Fe(ClO_4)_3$], all of the ferric iron (10^{-4} M) was rapidly reduced to the ferrous form and subsequently stabilized

by the tannic acid at a pH of 6.3. When the order of addition of the ferric iron and organic compound to the reactor was reversed, the iron was not reduced by the tannic acid, but formed a ferric hydroxide precipitate.

In the preliminary studies, the S-medium could stabilize only 1.5 mg/l of iron at a neutral pH. In order to increase the stabilization capacity of the S-medium, additional organic chelating agents were used. Cysteine, citric acid and gallic acid were used in different phases of the study. When adding iron to the S-medium, a freshly prepared solution of $FeCl_3$ (10^{-2} M) was added prior to adjusting the pH of the medium to the pH of the test condition. The stock solutions of the iron-organic complex (cysteine, citric acid, gallic acid) were prepared by adding $FeCl_3$ to a solution containing 10^{-2} M of the organic compounds. The pH was then adjusted to the test condition and filtered through either a PM-10 membrane (polar ultrafiltration membrane with a 10,000 molecular weight cut-off) or a 0.45 μm membrane filter. The iron passing these filters was considered to be the "total soluble iron."

5. Distribution of Iron Deposits in the *Sphaerotilus*

The determination of the distribution of iron across the sheath and cell wall of the iron-organic complex inhibited *Sphaerotilus* would be helpful in understanding the mechanism for the adsorption of iron by this organism. Some other characteristics of the iron adsorption, i.e., kinetic of the reaction, effect of the concentration of cell and organic complexing agents, were also studied. The procedures for this study were set as follows:

Determination of the Distribution of Iron in *Sphaerotilus*

(1) Sterilize 500 ml of S-medium in a 1000 ml Erlenmeyer flask.

(2) Inoculate the medium with 10 ml of the log growth phase culture of *Sphaerotilus*.

(3) Set the flask on a shaker.

(4) After 9 hours of incubation, determine the cell concentration.

(5) Add 20 mg/l of iron-cysteine complex in the culture; both culture and iron-complex solution are adjusted to pH 6 before they are mixed.

(6) Set the flask on a shaker for two hours.

(7) Analyze the sample as per the flowsheet in Figure 7.

As shown in the flowsheet, the sample was subjected to a sequence of centrifugations at 6,000 rpm, 10,000 rpm and 10,000 rpm (Servall Superspeed Centrifuge, SS-34 rotor) to wash out the residual soluble iron complex and remove the outermost layer of slime on the *Sphaerotilus*. Then, the sheath of *Sphaerotilus* was isolated from the precipitate by the method described by Romano and Peloquin (1963). The washed cell mass was suspended in 0.03 M tris(hydroxymethyl)aminomethane (pH 8.0). Tetrasodium EDTA and lysozyme were added at final concentration of 500 mg/l and 125 mg/l, respectively. This suspension was incubated at 37°C for 45 min. At this time sodium dodecyl sulfate was added to give a final concentration of 0.01 M, incubation was continued for an additional 30 min. This procedure resulted in the complete lysis of cells within the sheath and leaves the sheath undissolved for further analysis. The sheath was recovered by centrifugation at 15,000 rpm for 20 min in a Servall Superspeed centrifuge and was washed 3 times with distilled water. The sheath material was hydrolized

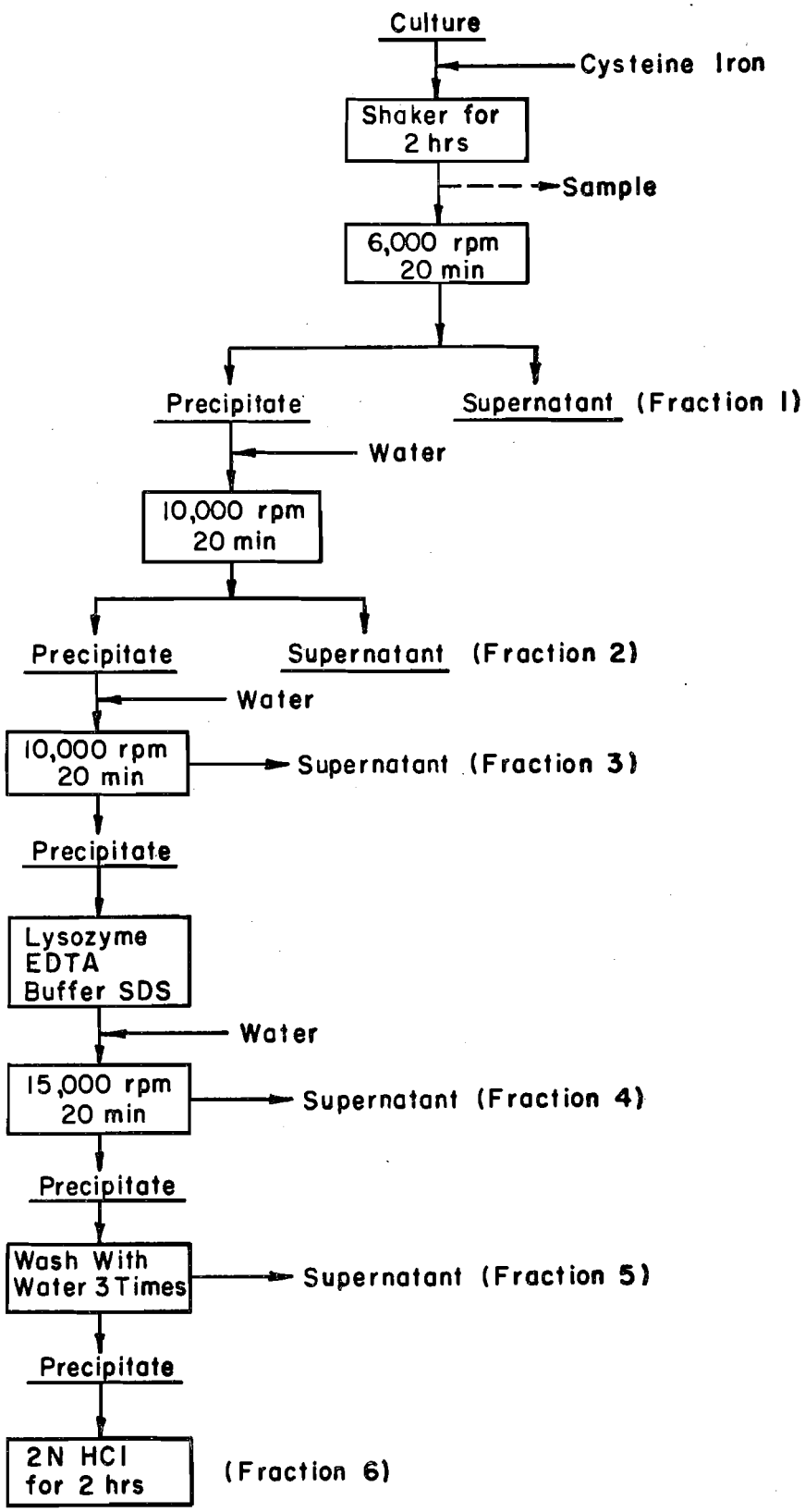


Figure 7. The Analytical Procedure for the Determination of the Distribution of Iron in *Sphaerotilus*

with 2N HCl at 100°C. A 0.45 μm membrane was used to filter the samples collected in each step. The total iron in both filtered and unfiltered samples was determined with the atomic absorption technique.

6. Analytical Techniques

Total iron and aluminum concentrations were determined with a Perkin Elmer Atomic Absorption Spectrophotometer Model 370. The water samples were acidified with 1 ml of concentrated nitric acid per 100 ml of sample and autoclaved at 121°C for 1 hour to solubilize the particulate matter. If the metal concentration exceeded 10 mg/l, an appropriate dilution was made to reduce the concentration to 10 mg/l or less. In this range, the absorbance varies linearly with the metal concentration. Triplicate readings were taken on each sample.

The "total soluble iron" is defined as the iron in the sample passing through a PM-10 ultrafiltration membrane manufactured by the Amicon Corporation. The membrane was set in a static membrane test cell equipped with a magnetic stirring bar which rotated just above the membrane surface. Pressurized filtration was provided with nitrogen gas. Immediately after each filtration the membrane was removed and rinsed thoroughly with a 1 M citric acid solution to remove any deposited material. After the initial studies, it was found that the results obtained by filtering the sample with a 0.45 μm membrane were the same as with the PM-10. The 0.45 μm filter was used in subsequent analyses.

V. RESULTS AND DISCUSSION

1. *Sphaerotilus* Species

In order to obtain the *Sphaerotilus* organism, bulked activated sludge was produced at a laboratory activated sludge system. The normal sludge in this system when fed with sewage had a dark brown color and Sludge Volume Index (SVI) around 70. As the sewage in the feed was replaced with a synthetic medium and dechlorinated tap water, the color of the sludge changed from dark brown to light brown and then white. The SVI of the sludge increased from 70 to over 1000. Because the bulked sludge settled poorly and did not compact well in the secondary settling tank, large quantities of the sludge were lost in the secondary effluent. The concentration of suspended solids in the aeration basin mixed liquor decreased to less than 1000 mg/l even though the return sludge ratio of the activated sludge system had been increased to 3.0.

A significant shift in the microbial population was observed. Filamentous organisms from the bulked sludge were examined with the identification methods described previously. The population of *Sphaerotilus* increased rapidly and at last became the predominate species in the sludge.

A pure culture of *Sphaerotilus* was obtained by streaking the effluent from the bulked activated sludge system on solid medium and restreaking the isolated colonies of *Sphaerotilus* on fresh plates. The growth obtained on this solid medium was good and was characterized by outgrowths along the lines of inoculation which had rough edges and a wavy interlaced center. The pure culture was then transferred from the solid medium to S-medium and incubated at room temperature (23°C). After 9 hours of incubation, the culture was confirmed to be *Sphaerotilus*.

The organisms enclosed their rod-shaped cells in sheaths. False branching was found with some trichomes in the mixed culture. The Prussian Blue test showed that both organisms from the mixed culture and pure culture isolate oxidized ferrous iron to ferric form under aerobic condition and precipitated the ferric iron on their sheaths. Although the organisms from both mixed and pure cultures showed positive responses in sulfur deposit and lipid deposit tests, the organisms from the mixed culture contained more sulfur in the cells while the pure culture isolate contained more lipid.

The *Sphaerotilus* species isolated in this study was a gram-negative and catalase-positive organism. It can use either peptone or ammonium sulfate as a nitrogen source. The optimum amount of ammonium sulfate that can be added to replace peptone as the nitrogen source in S-medium was found to be around 25 mg/l. When the modified S-medium was supplemented with 4 ppb of vitamin B_{12} (cyanocobalamine), a substantial increase in growth rate was observed. However, no growth was obtained with sodium nitrate as the nitrogen source. Growth of the *Sphaerotilus* isolate was found to occur at temperatures up to 40°C, while no growth was observed at 10°C. The desirable pH range was found to be from 6.0 to 9.0. For the above evaluations, the growth rate was measured by the maximum oxygen consumption rate as determined by the Warburg manometric technique.

A *Sphaerotilus* species (*Sphaerotilus natans Kutzing* 15291) was obtained from the American Type Culture Collection (ATCC) to verify the identification technique. The ATCC species of *Sphaerotilus* was identified to have the same morphology and cytology as the isolated species.

When the results from this study are compared with that in the literature (Stokes, 1954; Farquhar and Boyle, 1971a, 1971b), they are rather close. A comparison of the isolated species with the ATC species and the literature reports is given in Table 6.

Table 6. A Comparison of the *Sphaerotilus* Isolate with ATCC Species and Literature Reports

Character	Isolated Species	ATCC Species	* Literature Reports
Colonies	Outgrowths with rough edges along the lines of inoculation	Outgrowths with rough edges along the lines of inoculation	(1) Outgrowths with rough edges along the lines of inoculation
Sheath	positive	positive	(1),(2) positive
Shape of cell	1.0 μm to 2.0 μm by 3.0 μm to 8.0 μm rod	1.5 μm to 2.0 μm by 3.0 μm to 8.0 μm rod	(1) 1.0 μm to 2.0 μm by 2.0 μm to 8.0 μm rod
Iron oxidation	positive	positive	(2) positive
Sulfur deposit	positive	positive	(2) positive
Lipid deposit	positive	positive	(1),(2) positive
Gram-stain	negative	negative	--
Catalase	positive	positive	--
Growth temperature	15°-40°C	10°-42°C	(1) 10°-40°C
Growth pH	6.0-9.0	5.5-9.0	(1) 5.5-9.0
Utilization of nitrate	negative	negative	--
Utilization of ammonia nitrogen	positive	positive	(1) positive

*Sources:

(1) Stokes (1954).
(2) Farquhar and Boyle (1971a, 1971b).

2. Respiration Pattern

The respiration rate of *Sphaerotilus* was measured by the oxygen consumption as determined by the manometric technique. Figure 8 shows an oxygen consumption curve for *Sphaerotilus*. The slope of the curve is the respiration rate of the culture. During the first six hours of the Warburg test the concentration of substrate compared to the cell concentration was relatively high. This was the log growth phase where organism mass was the predominate rate limiting factor for the respiration. Cells were highly active in synthesizing new cells and the new cells increased the respiration rate.

After the log growth phase, the concentration of substrate decreased to a lower level so that it became the rate limiting factor and the rate of cell synthesis for each cell, i.e., specific growth rate, began to decrease. Accordingly, the slope of the curve, i.e., the respiration rate, begins to decrease. The maximum respiration rate occurred during this period and the curve approximates a straight line. Since the respiration rate is directly related to the cell synthesis, the maximum respiration rate would be proportional to the maximum growth rate of the culture. It would be reasonable to use the maximum respiration rate to express the maximum growth rate of the organism on S-medium.

After 24 hours of respiration, the culture was filtered through a 0.45 μm membrane. TOC analysis of the filtrate showed that 54 percent of the organic carbon of the medium remained in the filtrate. A concentration of 105 mg/l of total suspended solids was found after 24 hours of growth. These suspended solids was presumed to be the concentration of cells in the

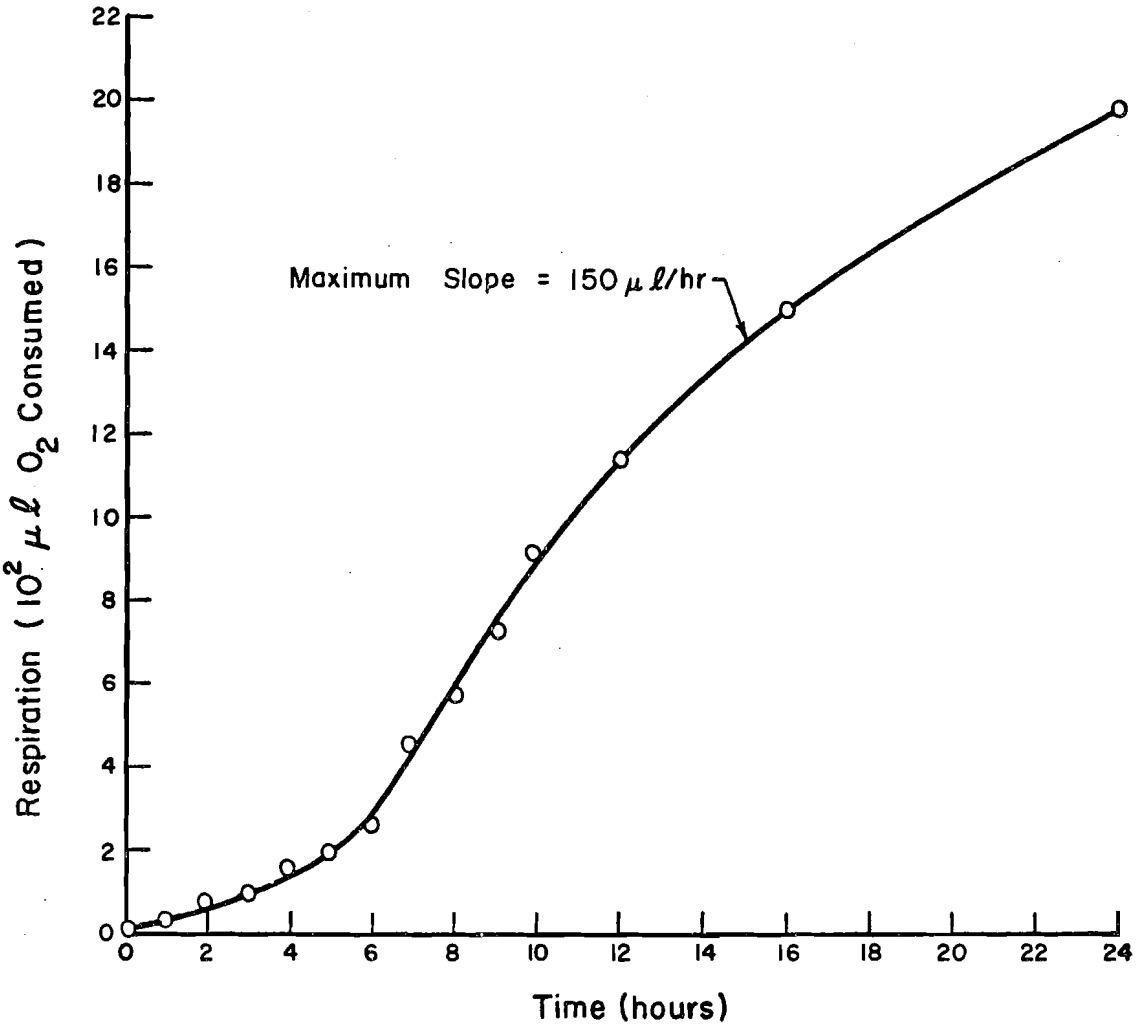


Figure 8. The Respiration Pattern of *Sphaerotilus* Isolate

culture. Assuming that 55 percent of the cell mass is organic carbon, an equivalent concentration of 58 mg/l of organic carbon would be present in the solid phase in the culture.

As Figure 8 shows, the total oxygen consumption of the batch of the culture is 2 ml of 0.089 m moles. It is assumed that the consumption of oxygen by the culture is for the oxidation of the carbon in the S-medium. The equivalent amount of organic carbon oxidized is 1.07 mg or 42.8 mg/l based on the volume of the culture.

Theoretically, the sum of the organic carbon in the solid phase and gas phase is the total conversion of the organic carbon in the S-medium during the 24 hours respiration period. The sum of the organic carbon in the solid phase and gas phase analyzed is 100.8 mg/l or 40.32 percent of the total organic carbon in the S-medium (250 mg/l). The result from the above calculation is close to the carbon loss calculated from the TOC analysis (46%). The above calculations show that a reasonable mass balance is obtained with the analytical methods employed in this study.

3. Iron-Organic Interaction

The inhibition of *Sphaerotilus* with a concentration of 25 mg/l of $FeCl_3$ in S-medium was reported by Waitze and Lackey (1959). Since the solubility of the ferric ions under neutral pH condition is very low, the inhibition of *Sphaerotilus* by the ferric ion seems to be unlikely. However, ferric ion may interact with organic matter to form soluble iron complexes. The inhibition of *Sphaerotilus* would be from the interaction of the organisms with the soluble iron complexes.

In the preliminary study, S-medium was found to stabilize only 1.5 mg/l of iron at neutral pH. As Figure 9 shows, the stabilizing capacity of S-medium for iron depends upon the amount of $FeCl_3$ added to the medium. Soluble iron in the S-medium increases with the increasing additions of $FeCl_3$ up to 1.5 mg/l as Fe. It begins to decrease with further addition of $FeCl_3$. When the addition of $FeCl_3$ exceeds 4 mg/l as Fe, all the iron precipitates. For the purposes of this study, increasing the stabilizing capacity of S-medium for iron by adding certain complexing agents to the S-medium was necessary.

After comparing several possible reagents, cysteine, citric acid and gallic acid were selected. Cysteine and gallic acid can reduce ferric iron to the ferrous form and retard the oxidation of this reduced ferrous iron while citric acid can chelate ferric iron and prevent the iron from precipitating. Some information regarding the interaction of these compounds with ferric iron have been found in literature. However, the conditions under which this information was obtained may not be applicable to this study. It will be necessary to verify the interaction between ferric iron and the selected complexing agents, i.e., cysteine, citric acid and gallic acid, before studying the inhibitory effect of iron on *Sphaerotilus*

The effect of reaction time on the stabilization of iron by these organic complexing agents was determined first. According to the report of Theis and Singer (1974), the procedure of the iron addition has a determinant effect on the formation of iron-organic complex. When iron salt was added to tannic acid solution, the reaction was complete before the first sample could be taken. A suitable amount of organic complexing agent was added in a flask containing 500 ml of S-medium and then $FeCl_3$ was added. In the preliminary

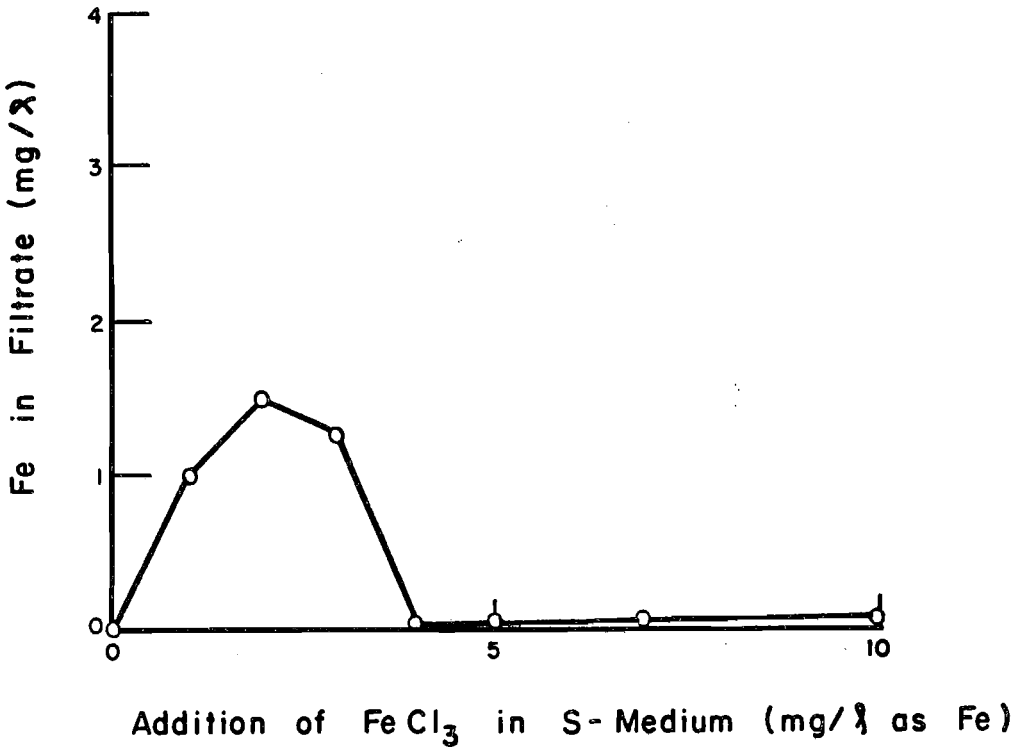


Figure 9. The Stabilizing Capacity of S-medium for Iron at pH 7.0

test it was found that the suitable amount of soluble iron for inhibiting the growth of *Sphaerotilus* was in the range of 5 to 20 mg/l. The amount of organic complexing agents corresponding to this range of soluble iron were approximately 2×10^{-3} M for cysteine and 0.5×10^{-3} M for citrate and gallic acid, or 244 mg/l for cysteine, 96 mg/l for citrate and 85 mg/l for gallic acid. It was decided that 200 mg/l of cysteine and 100 mg/l of citrate and gallic acid would be the concentration of complexing agents used in this study.

The pH of the stock solution of the organic complexing agents was measured at 1.91, 3.0 and 4.4 for cysteine, citrate and gallic acid, respectively. After these compounds were added separately to the S-medium, the pH of the media shifted to near the pH of the organic complexing agents. When the $FeCl_3$ solution (pH 1.7) was added, little change of pH was found. The flask containing the S-medium plus the organic complex and iron was set on a shaker. Samples were taken from the flask after a predetermined reaction period and immediately filtered through a PM 10 membrane (The samples for iron-cysteine reaction were adjusted to pH 3.0 with NaOH to precipitate ferric ion before the filtration).

Since the ferric ion concentration is around 0.5 mg/l at pH 3.0, all the iron in the filtrate is presumably in iron-organic compolex. As Figure 10 shows, all reactions are actually complete before the first sample is filtered through PM 10 membrane. This study indicates that when the iron is added to the medium according to the procedure described above, the interaction of iron with organic complexing agents will be complete in 5 minutes.

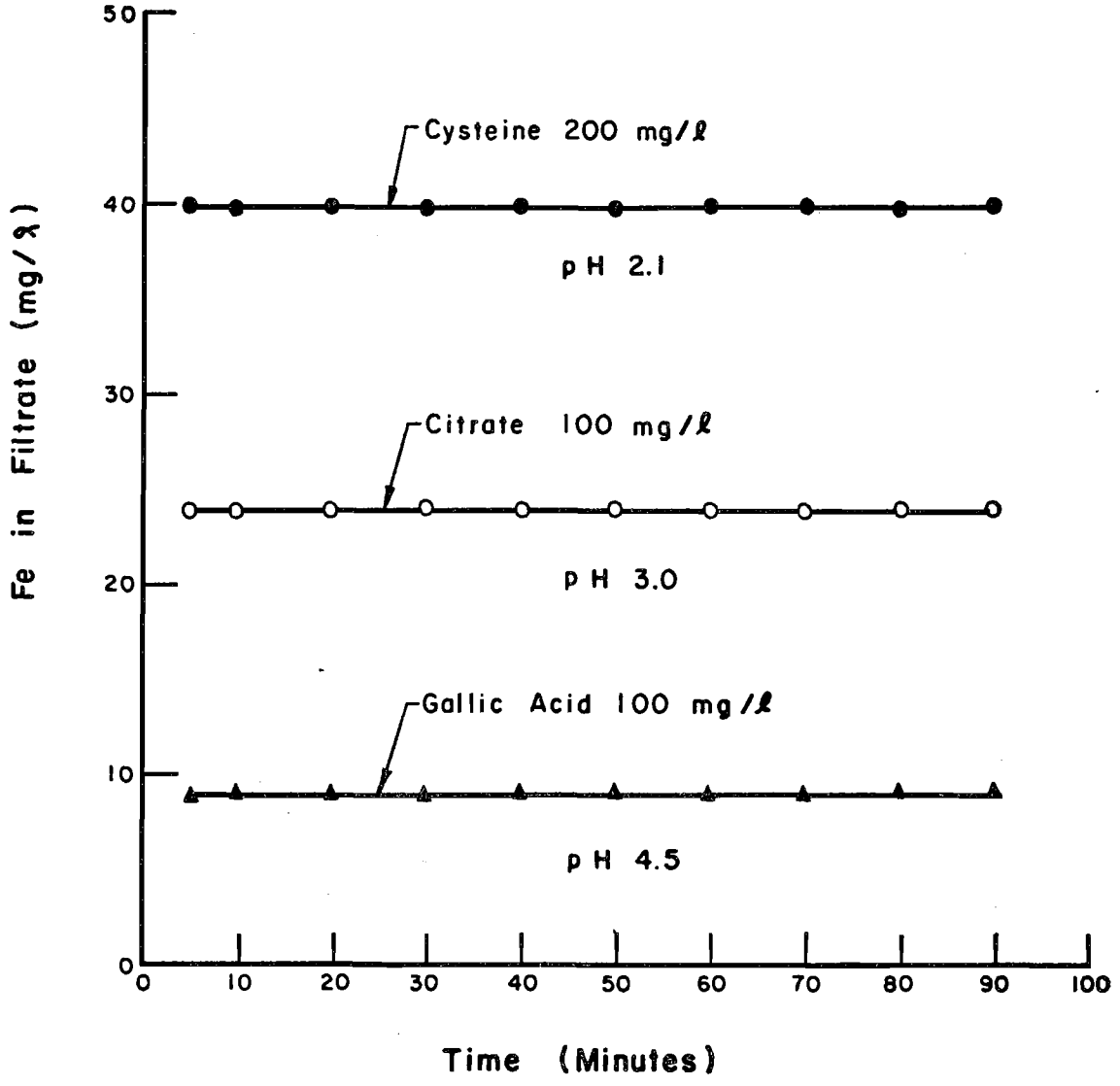


Figure 10. Effect of Reaction Time on the Stabilizing Capacity of Organic Compounds for Iron

A study of the stabilization capacity of the organic complexing agents for iron was conducted. With the addition of these complexing agents to the S-medium, high concentrations of soluble iron were stabilized at a neutral pH. The concentration of the soluble iron complexes increases with increasing addition of complexing agents in S-medium (see Figure 11). With the aid of this figure, any concentration of soluble iron complexes can be prepared by adding a suitable amount of complexing agent to the S-medium.

The effect of pH on the stabilizing capacity of the complexing agents for iron was determined. As discussed previously, 200 mg/l of cysteine and 100 mg/l of citrate and gallic acid were set as the experimental concentrations for this study. As Figure 12 shows, the stabilizing capacities of cysteine and citrate for iron decreases with increasing pH while the pH has very little effect on the gallic acid complex stability.

It has also been found that the iron-cysteine complex is not stable at a pH higher than 6.0. As previously discussed, the iron in the iron-cysteine complex is in the ferrous form. At high pH, ferrous iron was found to be destabilized from the complex and oxidized to ferric iron and then precipitated. The destabilizing rate of the complex was found to be pH dependent. The half-life of the complex ranges from 1 week at pH 6.0 to 1 hour at pH 7.0. For this reason, it was decided to maintain the pH of medium at 6.0 for evaluating the inhibitory effect of iron-cysteine complex on *Sphaerotilus* while maintaining a neutral pH for the other two complexes.

Because calcium and magnesium can also chelate on organic complexing agents, the presence of these metals in the medium may decrease the stabilizing capacity of citrate for iron. It was reported that a decrease of

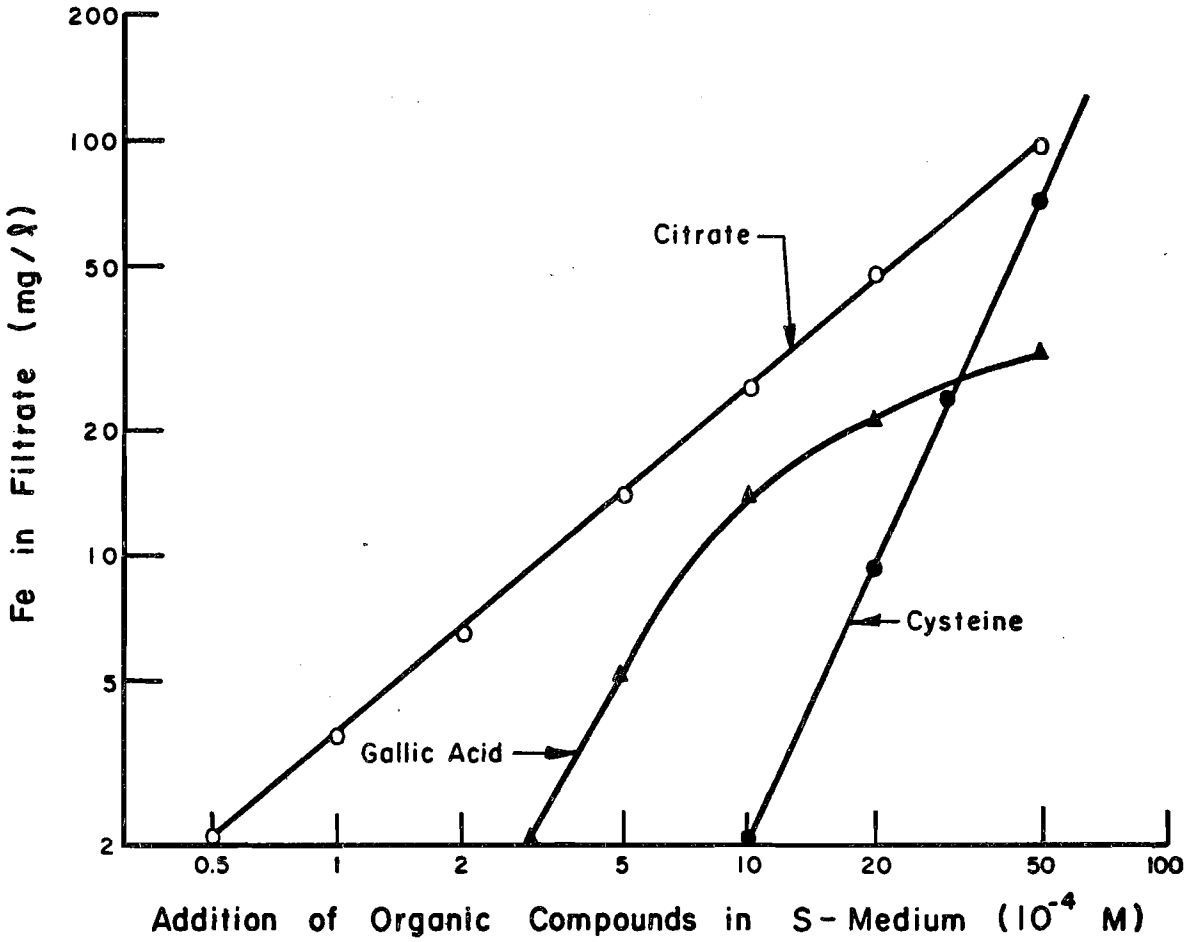


Figure 11. The Stabilizing Capacity of Organic Compounds for Iron at pH 7.0

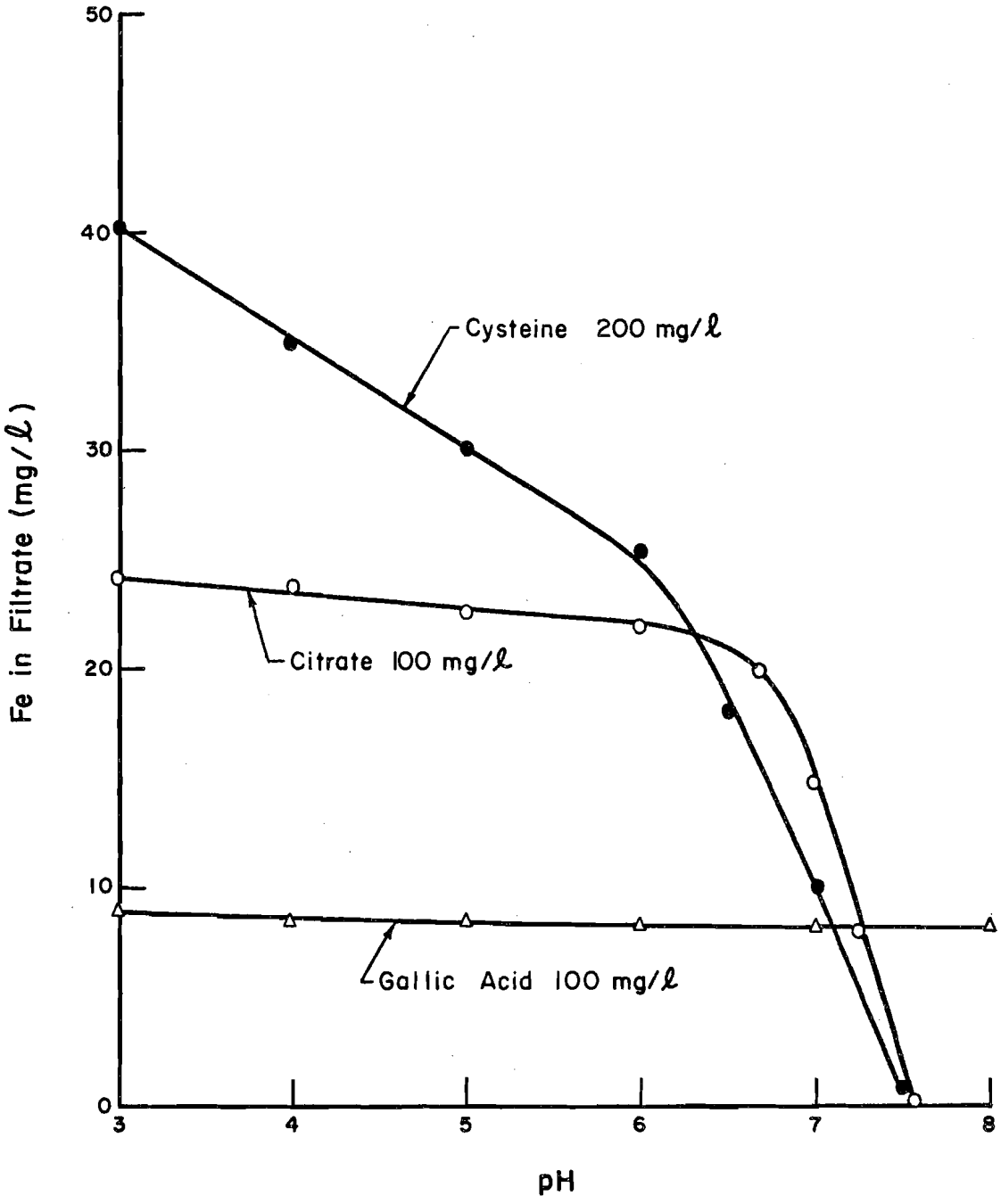


Figure 12. Effect of pH on the Stabilizing Capacity of Organic Compounds for Iron

80 percent of iron-citrate complex was found by adding 400 mg/l of calcium in a solution containing 4 mg/l of citrate and 0.1 mg/l of iron at neutral pH (Stumm and Morgan, 1970). However, little decrease in the stabilizing capacity of citrate for iron was observed when 100 mg/l of Ca^{++} and 50 mg/l of Mg^{++} were added in the S-medium containing 100 mg/l of citrate. In similar experiments with 200 mg/l of cysteine and 100 mg/l of gallic acid, no effect was found (Figure 13).

Because of the different experimental conditions, it is unrealistic to compare the result of this experiment to the literature reports. The constituents in the solution of the latter are much more complicated than the former. In addition to the higher concentration of citrate and lower addition of Ca^{++}, the presence of phosphate and other constituents in the medium must also be taken into account.

4. Inhibitory Effect of Iron Precipitates on *Sphaerotilus*

When an iron salt is added to the activated sludge system, iron will either interact with organic sludge or precipitate. It is desirable to know if iron precipitates have an inhibition effect on *Sphaerotilus*. Among the iron precipitates one needs to consider primarily fresh $Fe(OH)_3$, aged (5 days) $Fe(OH)_3$ and $FeCO_3$. Although iron phosphate may be among the precipitates, its amount will depend on the concentration of available phospahte. Because of the similarity of aluminum to ferric iron in physical-chemical properties, the inhibition effect of $Al(OH)_3$ on *Sphaerotilus* was also studied.

The iron precipitates and aluminum hydroxide were prepared for use in this study. Since ferrous ion is oxidized rapidly into ferric form under aerobic and neutral pH

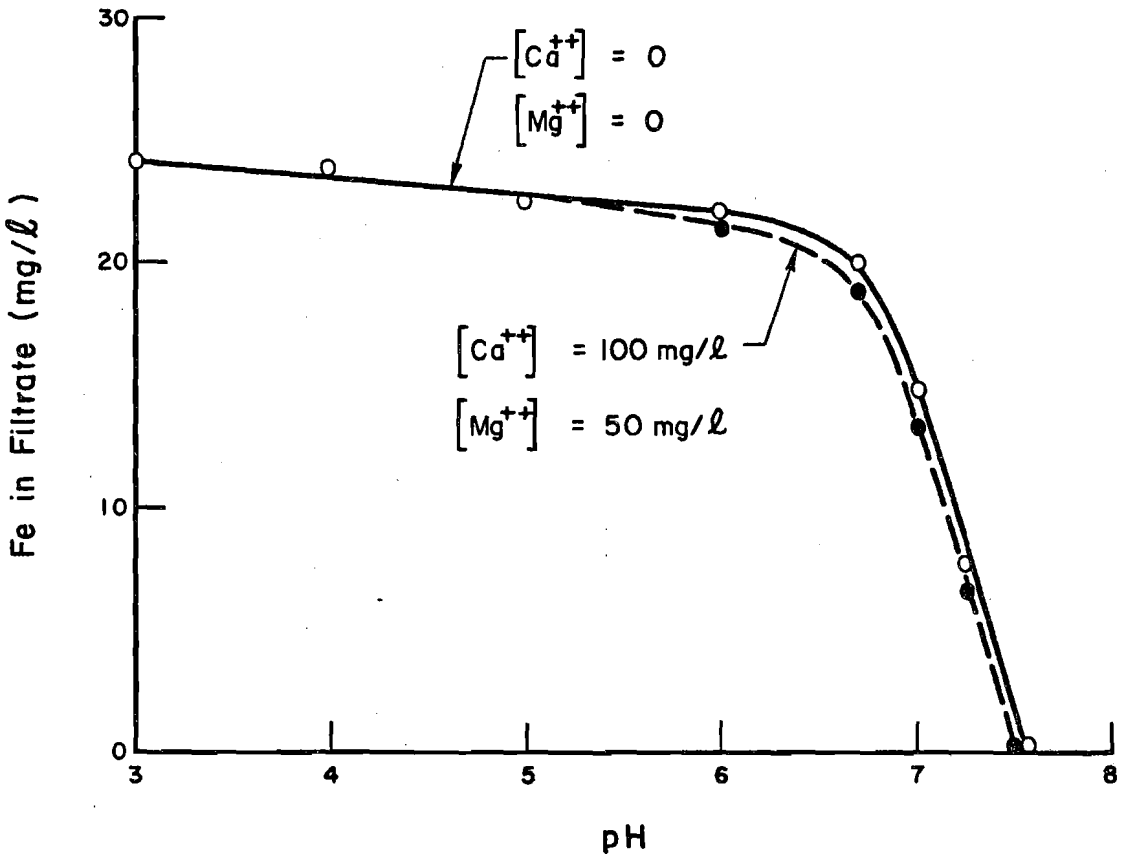


Figure 13. Effect of Calcium and Magnesium Concentrations on the Stabilizing Capacity of Citrate for Iron (Citrate = 100 mg/l)

conditions, the stability of $FeCO_3$ has to be considered. Part of $FeCO_3$ was found to be oxidized and precipitated as $Fe(OH)_3$ when the stock solution of $FeCO_3$ was prepared. This $Fe(OH)_3$ may precipitate on the surface of $FeCO_3$ particles and form a protection layer preventing $FeCO_3$ from further oxidation. Hence, the $FeCO_3$ particles actually were coated with a layer of newly precipitated $Fe(OH)_3$ and would have similar physical-chemical properties as fresh $Fe(OH)_3$ precipitate.

Iron precipitates were found to have very little inhibitory effect on *Sphaerotilus* (Figure 14). Among the iron precipitates studied, aged $Fe(OH)_3$ was found to be the most effective. However, it required 1000 mg/l of $Fe(OH)_3$ to reach 70 percent inhibition. The inhibitory effect was interpreted as the percentage reduction in maximum respiration rate of the *Sphaerotilus* culture monitored with the manometric technique.

After 24 hours of incubation, the inhibited cultures were examined microscopically. The organisms in the cultures inhibited with aged $Fe(OH)_3$ were coated with a layer of $Fe(OH)_3$ particles outside their sheath. The size of the particles was approximately 0.1 μm in diameter. The thickness of the coating varied and parts of the organism were not coated at all. The cultures inhibited with fresh $Fe(OH)_3$ and $FeCO_3$ were similar to each other in morphology as predicted in the previous discussion. The particles had been condensed to flocs and large parts of the organism were not coated with the particles. The culture inhibited with $Al(OH)_3$ was characterized by a rather thick uniform layer of $Al(OH)_3$ on the surface of *Sphaerotilus*. However, the $Al(OH)_3$ layer appeared to be poorly attached to the surface of the organism and also the layer was not very compact (Figure 15).

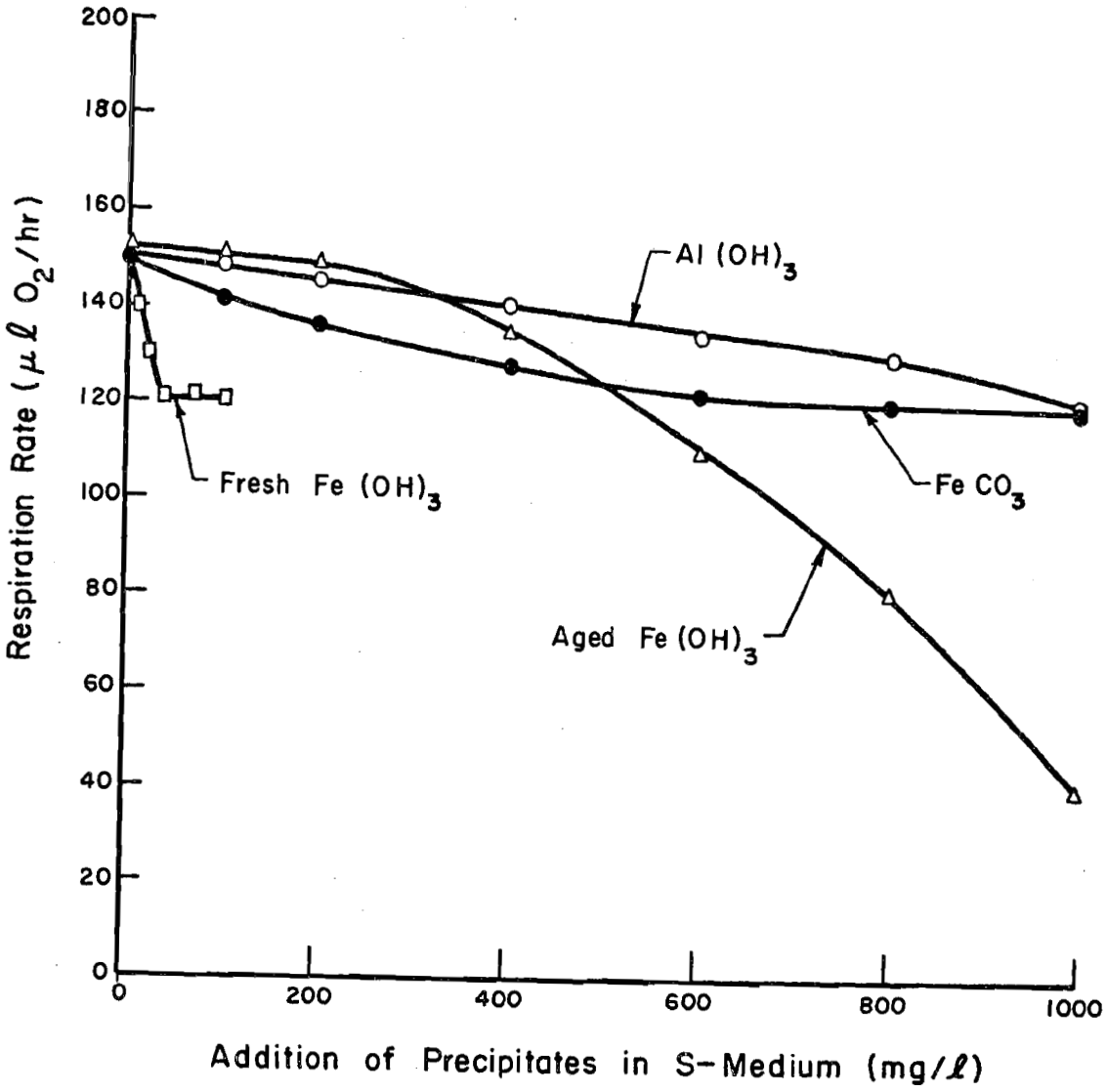


Figure 14. Inhibition Effect of Iron Precipitates on *Sphaerotilus* Isolate

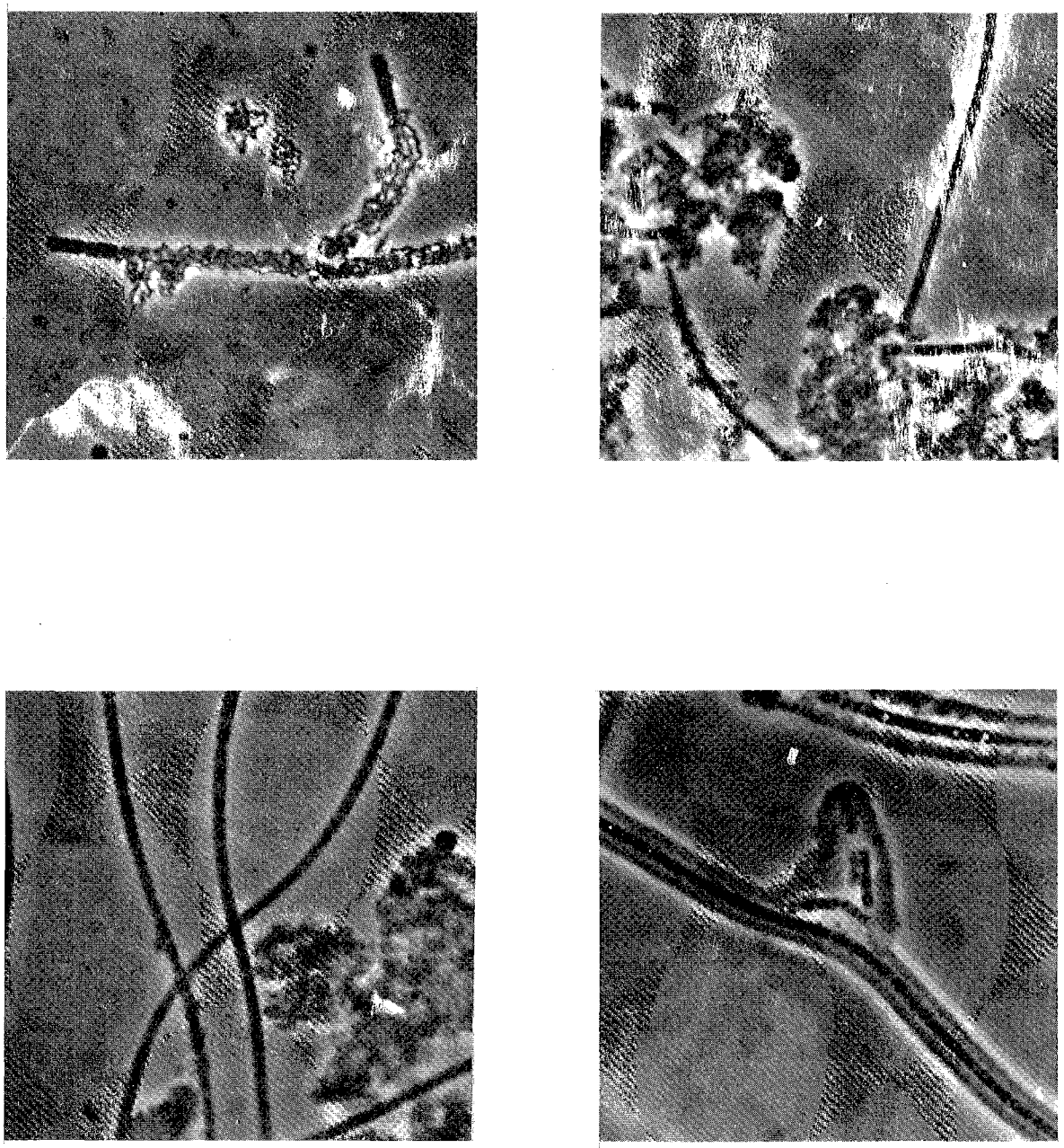


Figure 15. The Adsorption of Iron Precipitates on *Sphaerotilus* Isolate (x 1200)
(a) Aged $Fe(OH)_3$ (600 mg/l) (b) Fresh $Fe(OH)_3$ (600 mg/l)
(c) $FeCO_3$ (600 mg/l) (d) $Al(OH)_3$ (600 mg/l)

The mechanism for inhibiting *Sphaerotilus* with iron precipitates would be from the interaction of the iron precipitates with the organisms. The coating of iron particles outside the sheath of the organism would become a barrier to the transport of nutrients through the sheath and cell wall and hence inhibit the growth of the organism.

The effectiveness of the iron precipitates in inhibiting *Sphaerotilus* depends on the characteristics of the iron layer outside the sheath of the organism. Aged $Fe(OH)_3$ covered almost all of the organism while fresh $Fe(OH)_3$ and $FeCO_3$ covered a small portion of the organism. Consequently aged $Fe(OH)_3$ is more effective than fresh $Fe(OH)_3$ and $FeCO_3$ in inhibiting *Sphaerotilus*.

Although $Al(OH)_3$ forms a thick uniform layer outside the organism, it is poorly attached to the organism and also not very compact. These characteristics may be used to explain the ineffectiveness of $Al(OH)_3$ in inhibiting *Sphaerotilus*. By comparing the characteristics of the iron layer and aluminum layer on the organism, it is believed that the iron precipitates would form chemical bonds with the sheath of the organism. As the surface characteristics of the iron hydroxides and the interaction between the ferric iron and organic matter are considered, this assumption appears reasonable. Because the interaction potential of aluminum with organic matter is much less than ferric iron (Sillen and Martell, 1964), the poor attachment of the $Al(OH)_3$ on *Sphaerotilus* and its ineffectiveness in inhibiting the growth of the organism are predictable.

5. Inhibitory Effect of Soluble Iron on *Sphaerotilus*

In the above study, iron precipitates were found to have little inhibitory effect on *Sphaerotilus*. Therefore, the inhibitory effect of iron on *Sphaerotilus* was presumed to be from soluble iron. Since the

S-medium was found to stabilize only 1.5 mg/l of iron at a neutral pH, it was decided to add iron-organic complexes into the medium to increase the soluble iron concentration.

The iron-organic complexes used in this study were iron-cysteine, iron-citrate and iron-gallic acid. The characteristics of these iron complexes have been discussed previously. The iron in iron-cysteine and iron-gallic acid complexes are in the ferrous form whle the iron-citrate complex is in the ferric form. With regard to the effect of pH, the iron-cysteine is the least stable among these iron complexes while iron-gallic acid is the most stable.

The degree of inhibition was determined as the percentage reduction in the maximum respiration rate of the *Sphaerotilus* culture as measured with the manometric technique. As shown in Figure 16, when the pH of the medium was maintained at 6.0, the addition of 6 mg/l as Fe of iron-cysteine complex to the S-medium reduced the respiration rate of *Sphaerotilus* 90 percent. In order to achieve the same inhibition with the iron-citrate complex, 20 mg/l as Fe of the complex was required. In this test, the pH was maintained at 6.0 because the iron-cysteine complex is not stable at a higher pH. In another test, the effectiveness of iron-citrate and iron-gallic acid in inhibiting *Sphaerotilus* was compared. A neutral pH (6.7) was maintained in this test. The results showed that with an addition of 20 mg/l as Fe of the iron-citrate complex to the S-medium, the respiration rate of the *Sphaerotilus* was reduced 50 percent. With the same addition of iron-gallic acid complex, only a 10 percent reduction in respiration rate was observed.

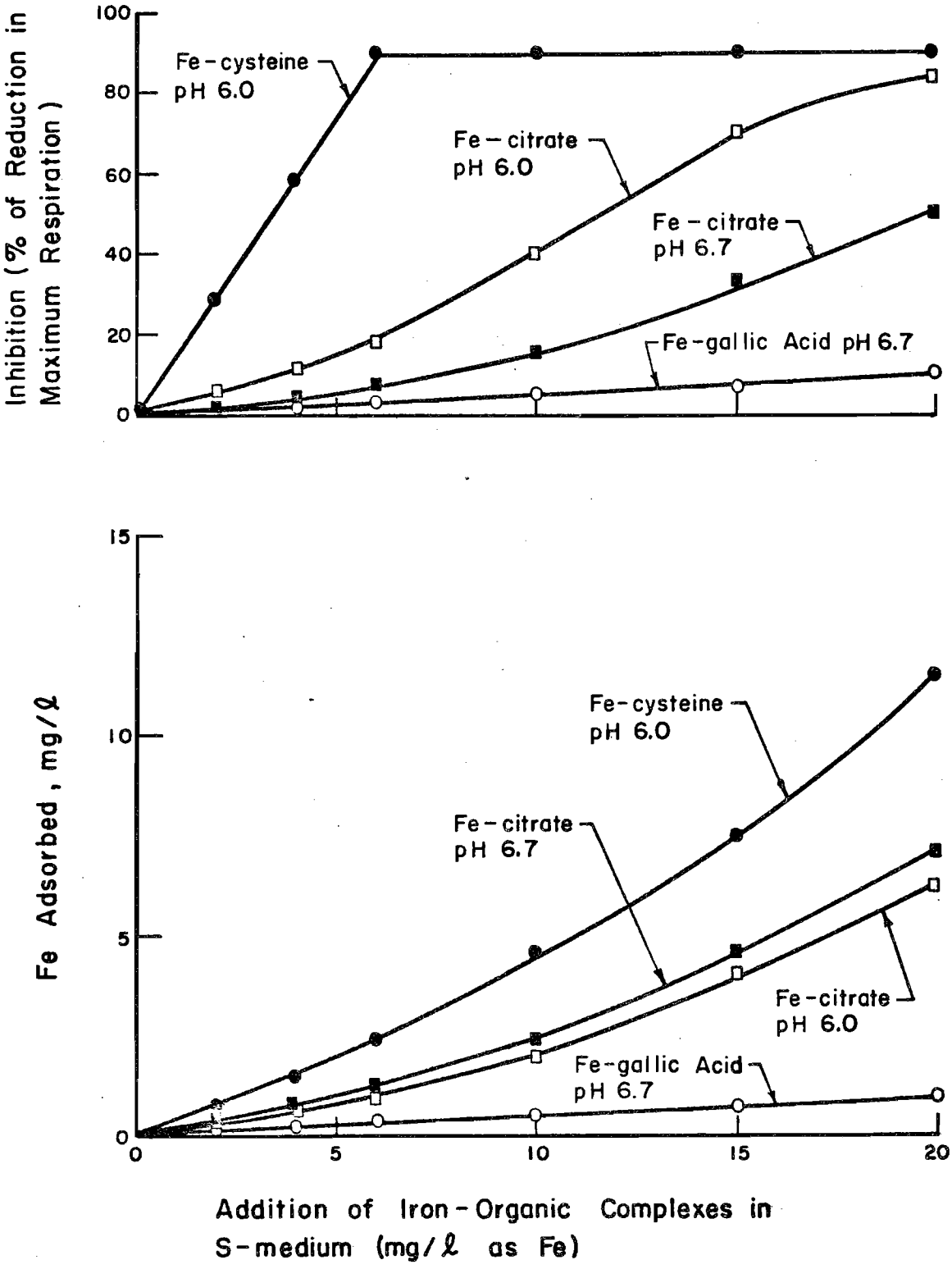


Figure 16. Inhibitory Effect of Iron-Organic Complexes on *Sphaerotilus* Isolate

After the respiration study was completed, the *Sphaerotilus* culture was removed from the respirometer flasks for soluble iron analysis and microscopic examination. The concentration of the soluble iron in the filtrate was determined with the atomic absorption technique. The difference in the soluble iron between the blank and the *Sphaerotilus* culture would result from the interaction of the iron complex with the organisms.

As shown in Figure 16, the adsorption of iron on *Sphaerotilus* increases with increasing additions of iron-organic complex. Accordingly, the inhibition effect of the iron-organic complex on *Sphaerotilus* increases with increasing adsorption of iron on this organism. A more relevant illustration of the relationship between the inhibition of *Sphaerotilus* and the adsorption of iron on this organism is shown in Figure 17. As this figure shows, the inhibition of *Sphaerotilus* is proportional to the amount of iron adsorbed on this organisms.

The iron inhibited cultures were examined microscopically with the aid of Prussian Blue Staining Technique. The organisms in the inhibited cultures were stained, producing a blue color. The uniform blue color indicated a layer of ferric iron on the organisms.

6. Mechanism of *Sphaerotilus* Inhibition by Iron

A possible mechanism for *Sphaerotilus* inhibition with iron is iron adsorption on the *Sphaerotilus*. The deposition of iron on the sheath of *Sphaerotilus* has been reported by several investigators (Pringsheim, 1949; Phaup, 1968; Mulder and van Veen, 1963). The accumulated iron may become a barrier to the transport of nutrients through the sheath and cell wall, and hence inhibit the growth of this organism.

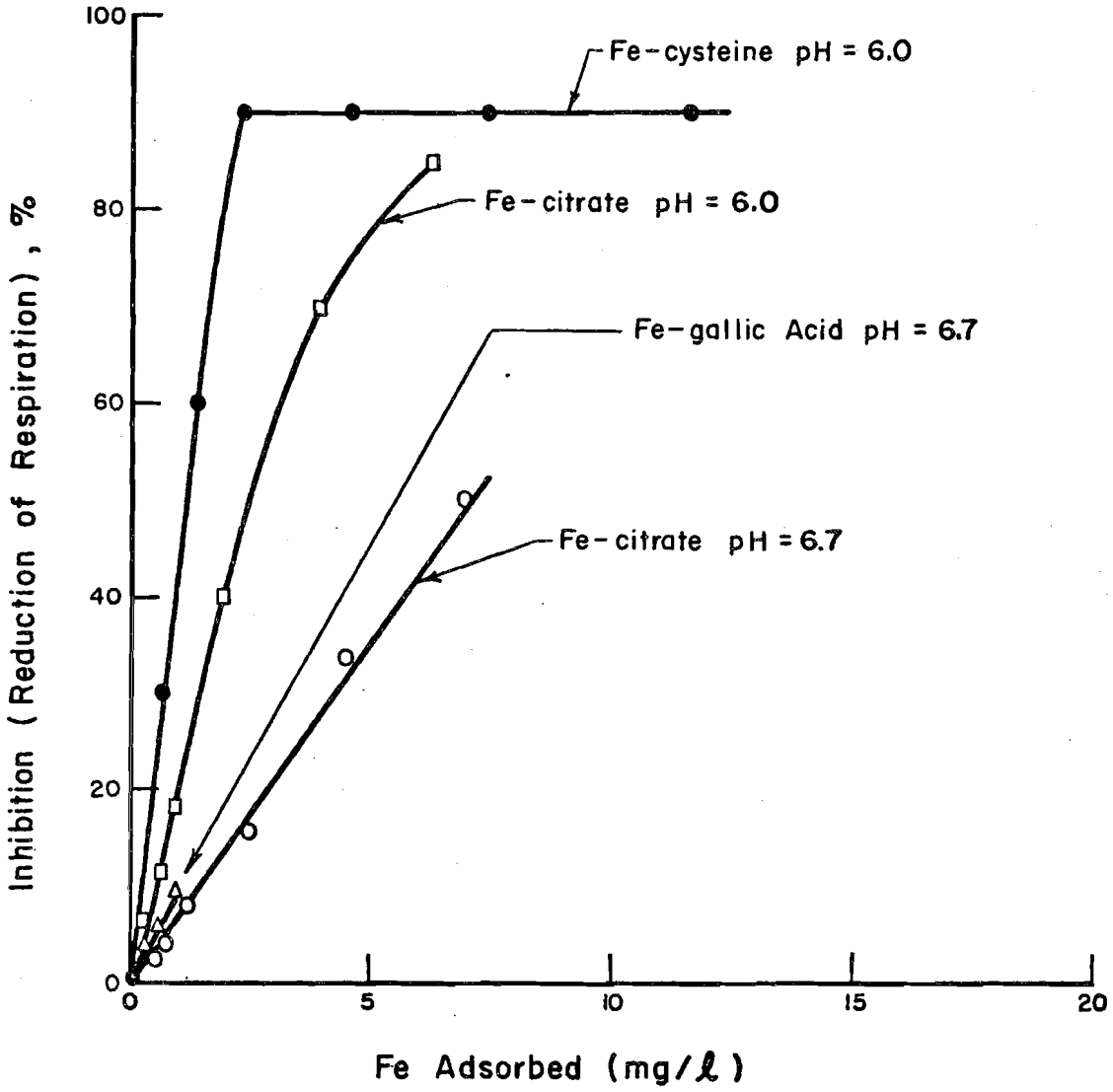


Figure 17. Relation between Adsorbed Iron and the Inhibition of *Sphaerotilus* Isolate

The inhibitory effects of soluble iron and iron precipitates on *Sphaerotilus* have been discussed previously. The study showed that both soluble iron and iron precipitates are adsorbed to the organisms. Microscopic examination showed that the inhibitory effects of different forms of iron could be related to the characteristics of the iron adsorbed to the organisms. For iron precipitates, aged $Fe(OH)_3$ coats a larger part of the organism than fresh $Fe(OH)_3$ and $FeCO_3$. Therefore, it is more effective in inhibiting the organism than the latter. Although $Al(OH)_3$ forms a thick uniform layer outside the organism, it is not effective in inhibiting the organism because it is poorly attached to the organism.

Soluble iron is adsorbed to the organism in a uniform layer so that it is more effective in inhibiting the organism than the iron precipitates. The analysis of soluble iron in the filtrate of the *Sphaerotilus* culture showed that the inhibitory effects are proportional to the amount of the adsorbed iron on this organism (Figure 17). Figure 17 also shows that among the soluble iron complexes, ferrous forms are more effective in inhibiting the *Sphaerotilus* than the ferric form. A hypothetical mechanism will be discussed later to relate this observation to the distribution of iron on *Sphaerotilus*.

7. The Distribution of Iron in *Sphaerotilus* Inhibited with Iron-Organic Complexes

As discussed previously, the adsorption of iron on *Sphaerotilus* is the main mechanism for the inhibition of this organism. The accumulated iron becomes a barrier to the transport of nutrients through the sheath and cell wall and hence inhibits the growth of the organism.

The characteristics of the iron adsorbed on *Sphaerotilus* are useful in explaining the difference in inhibition between the various forms of iron. The microscopic examination showed that the iron precipitates coat only part of the surface of the organism while the adsorbed soluble iron forms a uniform layer. This coincides with the observation that iron precipitates are less effective than soluble iron in the inhibition of *Sphaerotilus*.

However, the microscopic examination did differentiate between ferrous and ferric soluble iron inhibition. Figure 17 shows that the soluble ferrous iron was more effective than the ferric form. When the inhibited cultures were examined microscopically, no difference was observed between the cultures with ferrous and ferric forms. In both cases, all organisms were covered with a uniform layer of iron.

It is suspected that some iron may diffuse through the organism's sheath and be adsorbed on the cell wall when ferrous iron was employed. This is based on the assumption that the organism is more vulnerable to the adsorption of iron in the cell wall than on the sheath. Therefore, the ability of ferrous iron to diffuse through the sheath and adsorb on the cell wall makes the ferrous form of iron more effective in the inhibition of this organism. In an attempt to substantiate the above principal, an experiment was conducted to determine the distribution of iron in the sheath and cell wall of the iron-cysteine inhibited *Sphaerotilus*.

The experimental method has been described previously and is briefly reviewed below. The culture was washed three times to remove the residual soluble iron complex. The precipitated cell was lysed with lysozyme and EDTA in tris-buffer, and then with sodium dodecyl sulfate (SDS).

The sample was examined microscopically until the cells within the sheath completely disappeared (Figure 18). After the cell was lysed, the iron released would be chelated by EDTA and dissolved in the liquid. The sheath was recovered by centrifugation and the supernatant was collected for iron analysis. All of the iron in the supernatant was presumably from the lysis of the cell.

The end precipitate of the centrifugation of the test sample was dissolved in 2 N HCl, and the iron in this solution is presumably from the sheath. The results of the analysis are given in Table 7.

There was a question about whether or not the EDTA dissolved some iron from the sheath. In order to answer this question, a blank test for the cell lysis process was conducted (lysozyme and SDS were replaced with distilled water). The result showed that only 0.3 mg/l of iron was found in the filtrate of the blank, while 4 mg/l of iron was found in the supernatant of lysed cells when the addition of iron-cysteine in the *Sphaerotilus* culture was 20 mg/l as Fe. The soluble iron in the blank was only 7.5 percent of the soluble iron in the test sample.

This analysis for the distribution of iron in the organism was conducted at three different levels of iron-cysteine complex addition, i.e., 10 mg/l, 15 mg/l, and 20 mg/l. The other conditions were constant. Table 10 shows that a rather significant amount of iron was released from *Sphaerotilus* when the cells were lysed. The trend of the data shows that for these test conditions, little change was found in the amount of iron associated with the cell wall (Fraction 4) while the amount of iron associated with the sheath (Fraction 6) increases rapidly with the increasing concentration of the iron-cysteine complex.

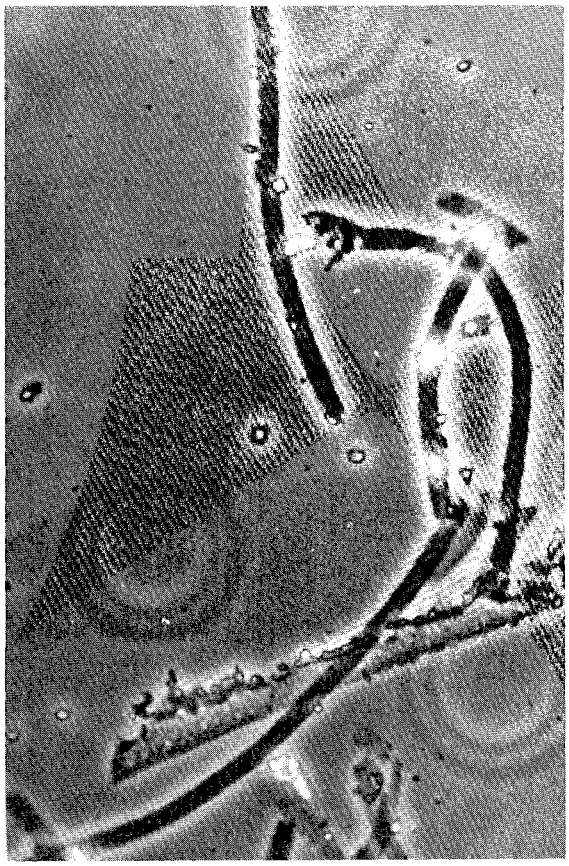


Figure 18. The Empty Sheath of *Sphaerotilus* Isolate after the Organism was Lysed with Lysozyme and SDS (x 1600)

Table 7. The Distribution of Iron in *Sphaerotilus* after Iron-Cysteine Was Added in the Culture*

Supernatants of Centrifugation	0.45 μm Filter	Fe-cysteine 20 mg/l 1 Fe mg	1 %	2 Fe mg	2 %	Average Fe mg	Average %	Fe-cysteine 15 mg/l Fe mg	15 mg/l %	10 mg/l Fe mg	10 mg/l %
6,000 rpm (Fraction 1)	Unfiltered	1.36	39.08	1.40	40.23	1.38	39.65	1.28	47.19	1.06	55.17
	Filtrate	1.28	36.78	1.29	37.07	1.29	36.92	1.20	44.12	1.03	53.45
10,000 rpm (Fraction 2)	Unfiltered	0.192	5.52	0.20	5.75	0.20	5.55	0.204	7.49	0.084	4.35
	Filtrate	0.067	1.92	0.07	2.01	0.068	1.97	0.11	4.04	0.076	3.97
10,000 rpm (Fraction 3)	Unfiltered	0.08	2.30	0.07	2.01	0.075	2.16	0.041	1.50	0.023	1.20
	Filtrate	0.016	0.46	0.018	0.52	0.017	0.49	0.016	0.59	0.011	0.58
15,000 rpm after Lysis of Cell (Fraction 4)	Unfiltered	0.54	15.52	0.54	15.52	0.54	15.52	0.49	17.98	0.37	19.40
	Filtrate	0.48	13.79	0.50	14.37	0.49	14.08	0.42	15.44	0.36	18.53
15,000 rpm Washing (Fraction 5)	Unfiltered	0.086	2.47	0.06	1.72	0.073	2.10	0.046	1.69	0.029	1.51
	Filtrate	0.016	0.46	0.015	0.43	0.016	0.46	0.015	0.55	0.024	1.25
Precipitate (Fraction 6)		1.22	35.06	1.20	34.48	1.21	34.77	0.652	23.97	0.35	18.10
Total Iron Recovered		3.478	99.95	3.47	99.71	3.478	99.75	2.713	99.82	1.916	99.73
Iron Added in the Medium-Culture		3.48		3.48		3.48		2.72		1.92	

* Cell concentration = 105 mg/l

This observation indicates that there is some maximum limit for the adsorption of iron beyond the inner surface of the sheath (Fraction 4). This limitation may be either the limited space between the inner surface of the sheath and the outer surface of the cell wall or the difficulty in transporting iron through the sheath after the sheath adsorbs a certain amount of iron.

When the data for the iron associated with the sheath (Fraction 6) are plotted versus the addition of iron-cysteine, they generate a straight line on log-log scale paper (Figure 19). This indicates that the adsorption on the sheath is similar to the general adsorption isotherm expressed by the Freundlich equation. Several points were selected on the extended line in Figure 19 to give supplementary data for projecting the trend of the iron distribution across the sheath and cell wall (Table 8). By employing the information in Table 8, the plot presented in Figure 20 shows the relationship between the adsorbed iron and the inhibition of *Sphaerotilus*.

In Figure 20, it is interesting to note that when the addition of iron-cysteine is below 10 mg/l, the iron associated with the cell wall is higher than that adsorbed on the sheath. As the addition of iron-cysteine increases beyond 10 mg/l, the situation reverses. For comparing the effectiveness of the adsorbed iron in *Sphaerotilus* inhibition, the information in the range below 6 mg/l is more useful. Beyond this point, the organism is completely inhibited. At the low concentrations of iron-cysteine, the inhibition of *Sphaerotilus* is found to be proportional to the iron associated with the cell wall. It is believed that in this range, the inhibition of *Sphaerotilus* is mainly from the iron associated with the cell wall. When this conclusion is applied to Figure 16, it becomes

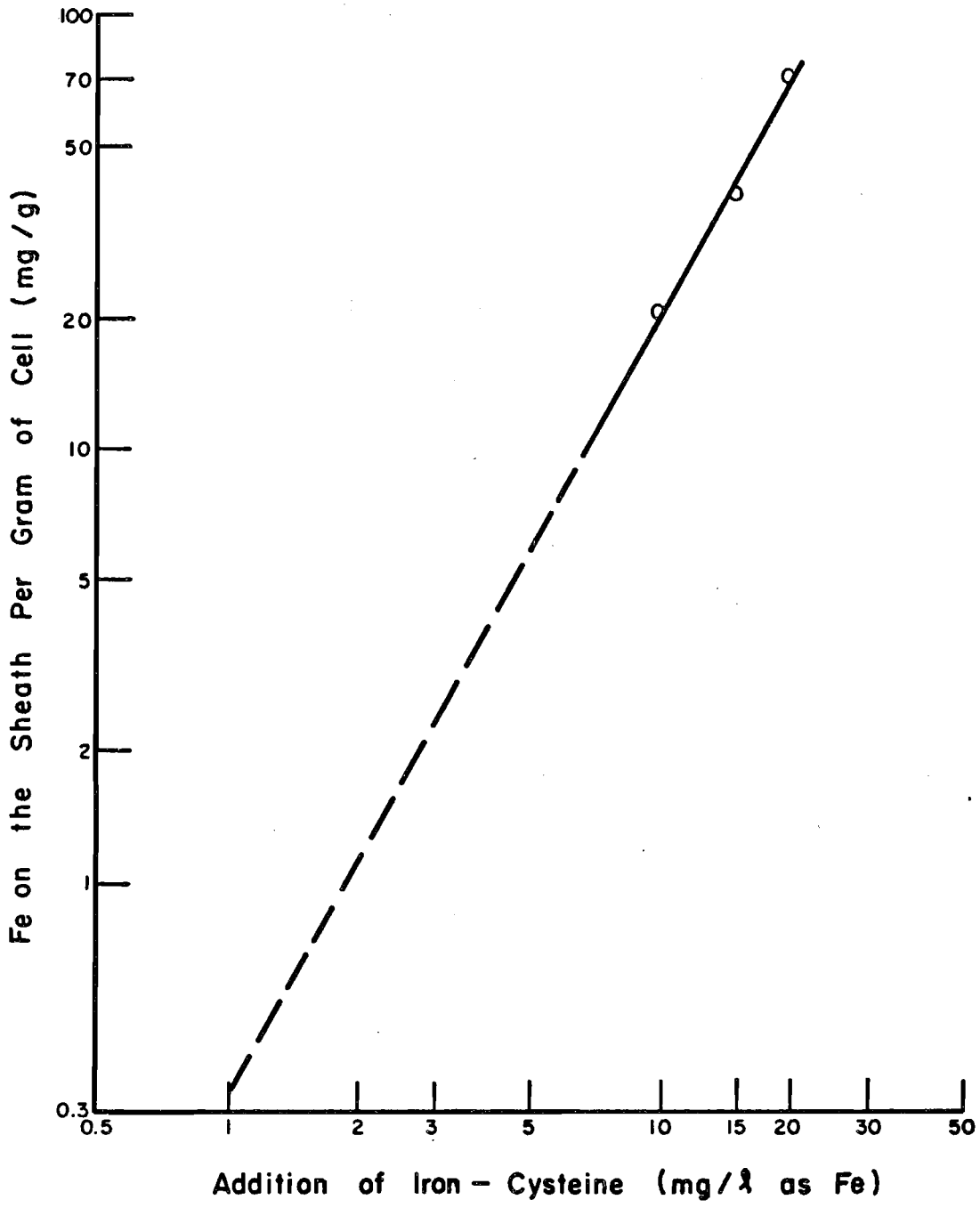


Figure 19. Iron Adsorption on the Sheath of *Sphaerotilus*

Table 8. The Relation of Inhibition and the Distribution of Iron in *Sphaerotilus* (Iron-Organic Complex = Iron-Cysteine)

Addition of Iron-Cysteine as Fe mg/l	Inhibition[2] %	Total[3] mg/l	Adsorbed Iron mg/g cell[4]	Fe on Sheath mg[5]	Fe on Sheath mg/g cell[6]	Fe from Cell mg[7]	Fe from Cell mg/g cell[8]
20	90	11.6	110	1.21	72	0.49	29.16
15	90	7.5	74.4	0.652	39	0.42	25.0
10	90	4.6	44.0	0.35	21	0.36	21.4
6	90	2.4	22.6		8*		14.6**
4	60	1.5	14.3		4*		10.3**
2	30	0.7	6.5		1.1*		5.4**

Volume of sample = 160 ml

Cell concentration = 105 mg/l

Cell mass = 16.8 mg

[2] and [3] from Figure 16

[4] = [3] x 0.16 ÷ 0.0168

[5] from Table 7 (Fraction 6)

[6] = [5] ÷ 0.0168

[7] from Table 7 (Fraction 4, filtrate)

[8] = [7] ÷ 0.0168

* from extended curve in Figure 19

** = [4] - *

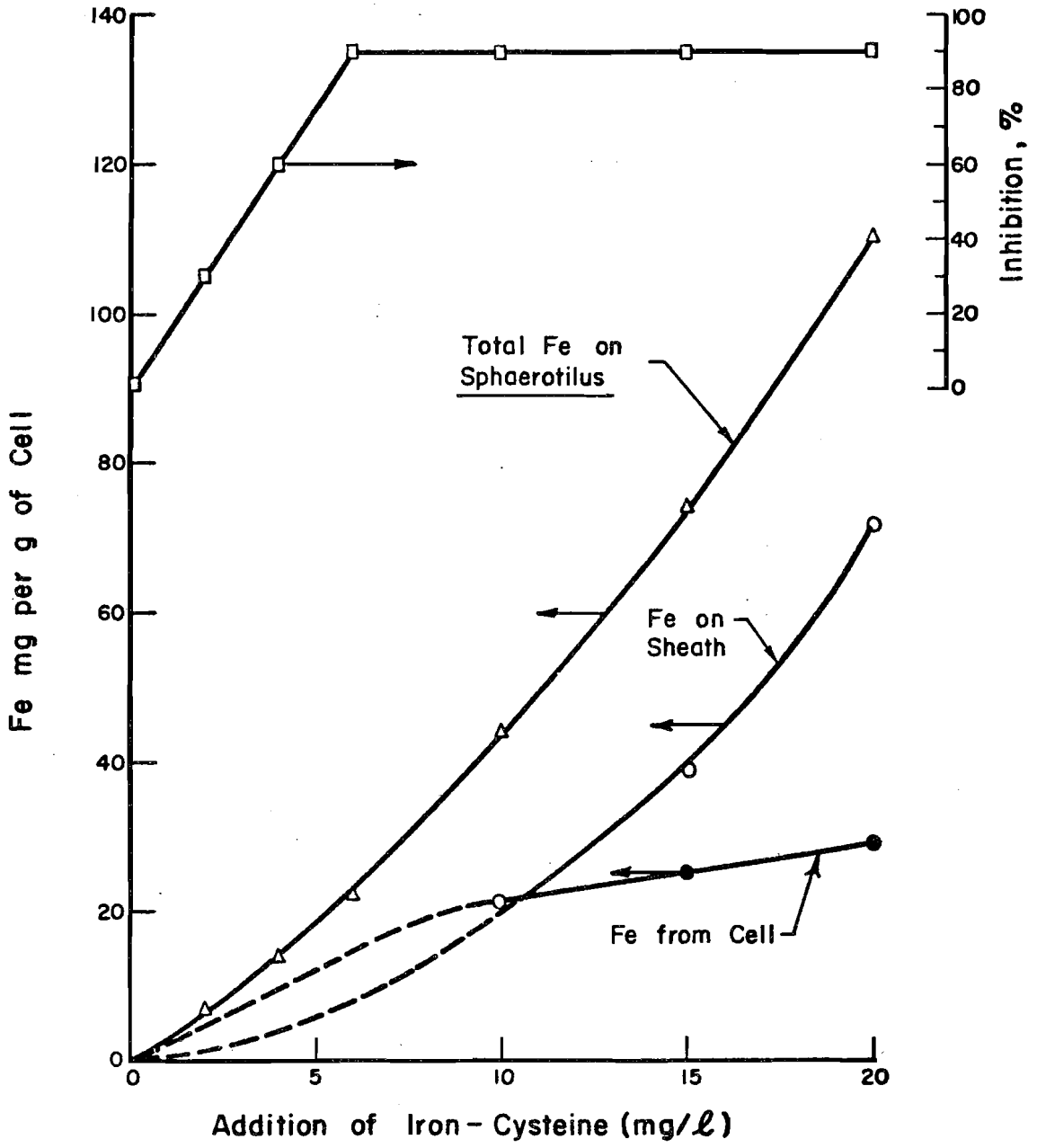


Figure 20. The Distribution of Iron on *Sphaerotilus* and Its Inhibitory Effect

more convincing. In Figure 16, both iron-cysteine and iron-gallic acid complexes are in the ferrous form and have the same pattern in *Sphaerotilus* inhibition. At low concentrations of iron-complexes, the curves show constant slopes for the inhibition and near constant slopes for adsorbed iron. The iron-citrate was in the ferric form and its curves show a different pattern; increasing slopes with increasing iron-citrate concentration.

Because the iron adsorption pattern for iron-citrate was similar to that for the iron adsorbed by the sheath when iron-cysteine was the inhibitor, it is presumed that most of the adsorbed iron from iron-citrate complex would be held on the sheath. This discussion leads to the following conclusion: With iron-organic complexes in ferrous form, i.e., iron-cysteine and iron-gallic acid, significant amounts of iron will be transported through the sheath and be retained either inside or outside the cell wall. With the ferric form, i.e., iron-citrate, most of the iron will adsorb on the sheath. Because *Sphaerotilus* is more vulnerable to the adsorption of iron on its cell wall than on its sheath, the ferrous form is more effective than the ferric form in inhibiting this organism.

8. Some Other Characteristics of the Adsorption of Iron by *Sphaerotilus*

As shown previously, both soluble iron and iron precipitates are adsorbed by *Sphaerotilus*. The adsorption of iron on the organism is the main mechanism for the inhibitory effect of iron. The effectiveness of the inhibition depends on the characteristics of the iron adsorbed by the organisms. The adsorbed soluble iron forms a uniform layer on the organism while the iron precipitates cover only part of the surface of the organism.

Therefore, soluble iron is more effective than the iron precipitates in *Sphaerotilus* inhibition. The ferrous form of iron-organic complexes can diffuse through the sheath and deposit on the cell wall while with the ferric form of iron-organic complex, most of the iron deposits on the sheath of the organism.

It is desirable to know what physical-chemical forces are involved in this adsorption of iron. An analysis of some of the characteristics of the iron adsorption, ie., kinetic of the reaction, effect of the concentration of cells and organic complexing agents, would be helpful in understanding the mechanism for the iron adsorption by *Sphaerotilus*.

Kinetic of the Iron Adsorption

In order to evaluate the kinetics of adsorption, a concentration of 20 mg/l of iron-organic complexes was added to a flask containing the *Sphaerotilus* culture. The flask was placed on a shaker. Samples were taken from the flask and immediately filtered through a 0.45 μm membrane according to a designated schedule. The iron concentration in the filtrate was measured to determine the reduction of soluble iron by the *Sphaerotilus* culture. As Figure 21 shows, the adsorption was rapid with the iron-cysteine complex. With cell concentration of 105 mg/l and an initial iron complex concentration of 20 mg/l as Fe, 80 percent of the reaction was complete within 5 minutes. Although the adsorption rate was slower for iron-citrate, 70 percent of the reaction was complete in 30 minutes. After approximately two hours, no further reaction was observed.

Both soluble iron and iron precipitates were adsorbed by the *Sphaerotilus*. Among the iron precipitates, aged $Fe(OH)_3$ and $Al(OH)_3$ have zero point of charge at 8.5 and 9.1, respectively.

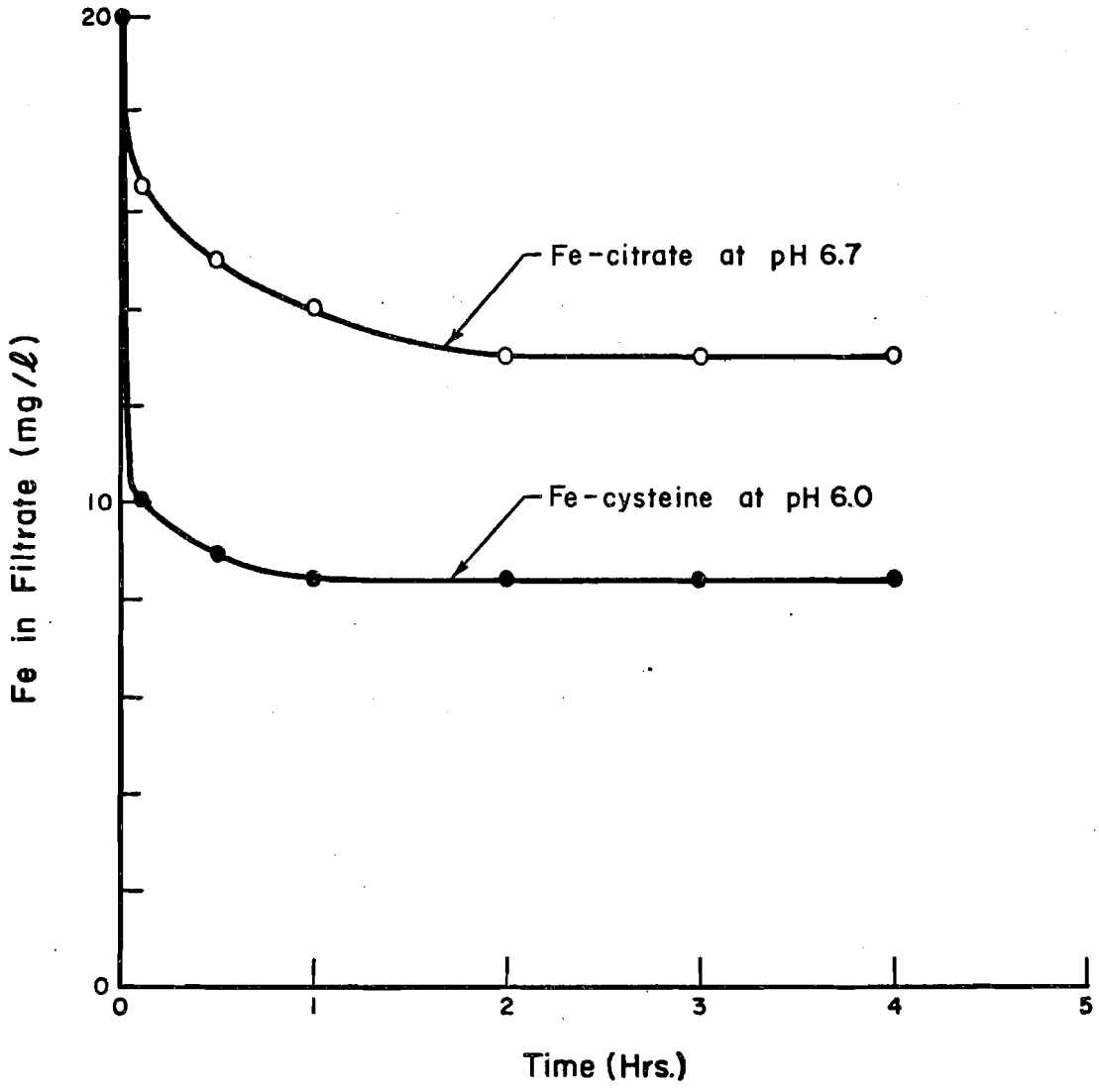


Figure 21. The Adsorption Rate of Fe on *Sphaerotilus* (cell = 105 mg/l)

Therefore, at a neutral pH, the surface charge of these two compounds would be positive. Since aged $Fe(OH)_3$ and $Al(OH)_3$ were positively charged under the experimental condition in this study, *Sphaerotilus* was presumably carrying a negative charge for this adsorption to occur.

The iron-cysteine complex and some species of iron-citrate complexes also are positively charged. The adsorption of these soluble iron complexes by *Sphaerotilus* would be the result of the attraction of these iron complexes by the negatively charged *Sphaerotilus* surface.

The charge condition on the surface of *Sphaerotilus* and the species of iron complexes has a significant effect on the kinetics of the iron adsorption. At the beginning of the reaction, the surface of the organism is unoccupied and the attracting force of the electric charge on the surface is at its highest. Therefore, the reaction is rapid. As some of the surface has adsorbed the iron, the attracting force of the charge decreases and hence the reaction rate decreases.

Because the charge condition is different for the iron-cysteine complex and iron-citrate complex, the reaction rate of iron adsorption is different for these two iron complexes. The iron-cysteine complex has two net positive charges. The iron-citrate has three forms in equilibrium, i.e., FeH_2L^+, FeHL and FeL^- (L = citric anion residual), and at neutral pH most of it is in the FeL^- form. Because the FeL^- form is repulsed by the negative charge on the surface of *Sphaerotilus*, only the less predominate species are attracted to the surface of the organism. Consequently, the iron-citrate has a slower reaction rate than the iron-cysteine.

After completion of the soluble iron analysis, the filtrate was analyzed for the total organic carbon (TOC). The objective of this test

was to determine the fate of the organic complexing agent when the iron was adsorbed by the microbial mass. The results are shown in Table 9. When citrate or cysteine, or their iron complex, was added to the *Sphaerotilus* culture, the TOC did not change during the four hours over which the test was conducted. Even though significant amounts of iron were adsorbed by the microorganisms, there was no significant change in the TOC. Therefore, it appears that after the iron is adsorbed, the organic counterpart is released back into solution.

Table 9. TOC Analysis Before and After the Iron Adsorption Reaction

Samples	Compounds added in *Sphaerotilus* Culture mg/l	TOC in Filtrate at Zero Time mg/l	TOC Filtrate in after 4 Hours of Reaction mg/l	TOC Reduction in Filtrate mg/l
1	S-medium	384	291	93
2	500 mg/l Glucose	334	274	60
3	100 mg/l Citrate	175	170	5
4	100 mg/l Citrate in Iron-Citrate Complex	175	174	1
5	200 mg/l Cysteine in Iron-Cysteine Complex	193	188	5
6	200 mg/l Cysteine	193	193	0
7	Blank	134	134	0

*Initial cell concentration = 105 mg/l
pH = 6.7 for samples 1, 2, 3, 4, 7
pH = 6.0 for samples 5, 6

Effect of the Concentration of *Sphaerotilus* and Organic Complexing Agents on Iron Adsorption

The procedure for evaluating the effect of the concentrations of microorganism mass on the adsorption of iron involved adding different amounts of the iron-organic complexes to two series of flasks that contained two different concentrations of *Sphaerotilus*. The flasks were placed on a shaker for two hours. Then samples were filtered through 0.45 μm membrane. The soluble iron concentration was measured to determine the quantity of soluble iron removed by adsorption on the microbial mass. The procedure used for this determination was the same as that used to study the effect of the organic complexing agents on the adsorption of iron. The variable was cell concentration rather than organic complexing agent.

Table 10. The Effect of the Concentration of Cell and Organic Complexing Agents on the Adsorption of Iron*

Cell Concentration mg/l	Organic Complexing Agents Concentration	Fe Addition in Fe-Organic Form mg/l	Iron %	Adsorption mg/g of Cell
105	200 (cysteine)	20	55	105
105	400 (cysteine)	20	48	91
105	100 (citrate)	20	31	59
105	200 (citrate)	20	22	42
53	200 (cysteine)	20	30	113
53	100 (citrate)	20	18	68

*pH = 6.0

The data in Table 10 show that the amount of soluble iron removed from the culture medium is a function of both the cell concentration and the complex form. The higher cell concentrations exhibited a higher percentage of iron adsorption. Also, the cysteine-iron complex was more readily adsorbed by the microbial mass. The citrate-iron complex was not adsorbed to the same degree. This is shown by the mg of iron adsorbed per g of cell.

The adsorption of iron by *Sphaerotilus* was also found to decrease with the increasing concentration of organic complexing agents. This effect is much less than the effect of cell concentration. The extent of this effect varies with different organic agents. When the concentration of the organic complexing agents was doubled, i.e., when the concentration of iron in the form of iron-citrate complex was kept at 20 mg/l level and the citrate concentration was increased from 100 mg/l to 200 mg/l, a 29 percent reduction in the iron adsorption was found. When the concentration of iron in the form of iron-cysteine complex was kept at 20 mg/l level while the concentration of cysteine was increased from 200 mg/l to 400 mg/l, a 13 percent reduction in the iron adsorption was observed.

The results of this study indicate that the surface area of the organism is the major factor for the adsorption of iron. The surface of the organism provides attracting electrical forces and the sites for the adsorption of iron so that the adsorption of iron increases with increasing cell concentration, i.e., the surface area of the organism. The concentration of the organic complexing agents is important in stabilizing the soluble iron. However, the increase of the concentration of the organic complexing agents in the solution increases the stability of the iron-organic complex and hence decreases the adsorption of iron by the organism.

9. The Proposed Theory for *Sphaerotilus* Inhibition by Iron

As discussed earlier, both soluble iron and iron precipitates are adsorbed by the *Sphaerotilus*. The soluble iron used in this study was in the form of iron-organic complex. The iron-citrate complex has three forms in equilibrium, i.e., Fe H_2L^+, Fe HL and Fe L^- (L = citric anion residual). The iron-cysteine complex has only one form with each molecule of the complex carrying two positive charges.

Among the iron precipitates, aged $Fe(OH)_3$ and $Al(OH)_3$ have zero point of charge at 8.5 and 9.1, respectively. Therefore, at a neutral pH, the surface charge of these two compounds are positive. Because the iron-cysteine complex, aged $Fe(OH)_3$ and $Al(OH)_3$ were positively charged under the experimental condition in this study, *Sphaerotilus* is presumably carrying a negative charge. Based on these observations, an electrical double layer on the surface of *Sphaerotilus* can be proposed as illustrated in Figure 22.

An analysis of the charge condition on the surface of *Sphaerotilus* would be helpful in understanding the mechanism of iron adsorption. Under the influence of a surface charge, the pH on the surface of *Sphaerotilus* will be different from that in the bulk solution. H^+ will be attracted to the surface of the organism, or more precisely, close to the rigid solution boundary. The pH outside a negatively charged surface may be estimated by Equation (9)(Stumm and Morgan, 1970).

$$\text{pH surface} = \text{pH bulk} - 0.87 \qquad (9)$$

Based on this equation, the pH on the surface of *Sphaerotilus* would be 5.83 when the pH of the bulk solution is 6.7.

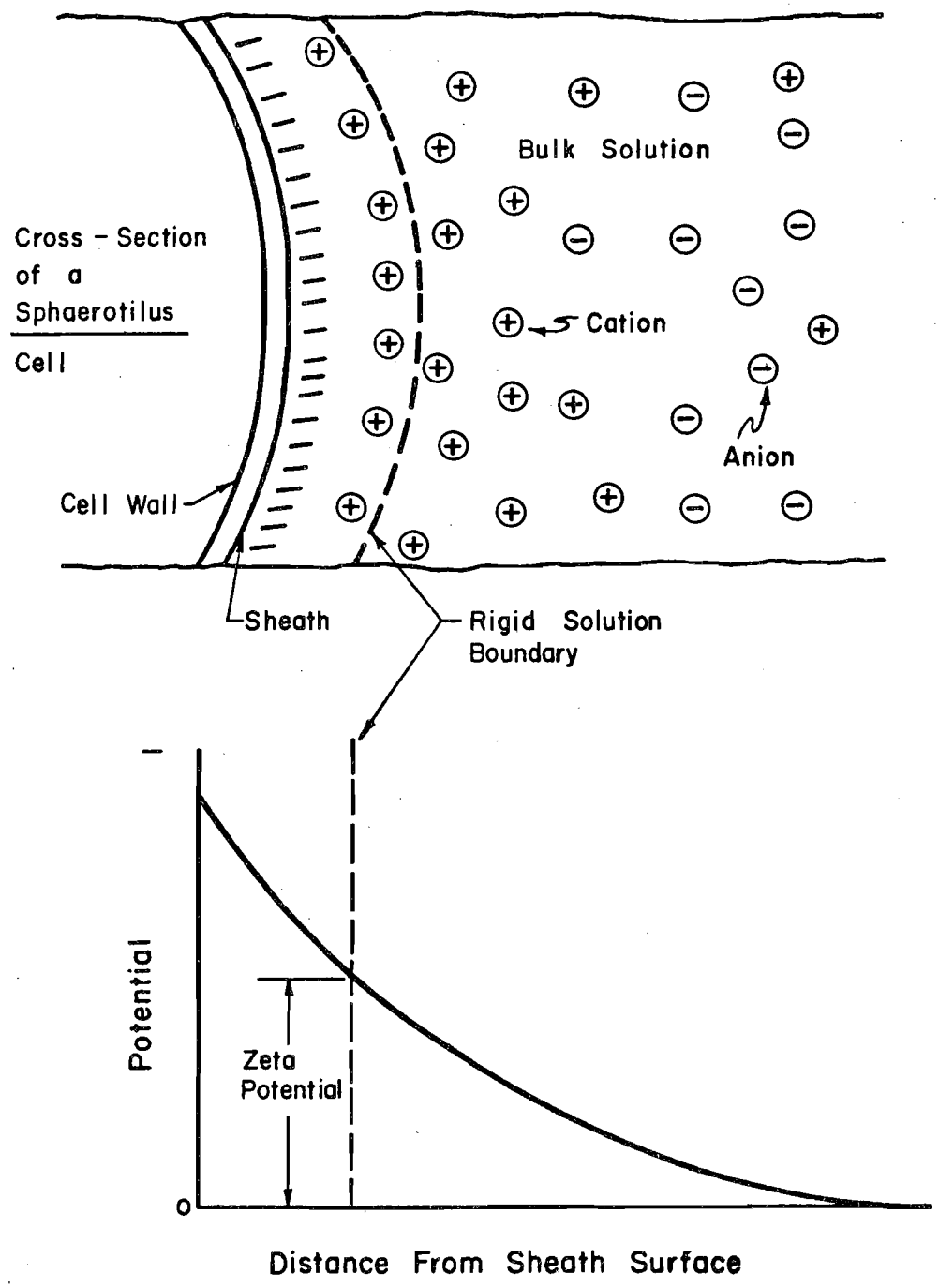


Figure 22. The Hypothetic Electrical Double Layer at Surface of *Sphaerotilus*

The negative charge and specific pH on the surface of *Sphaerotilus* plan an important role in the adsorption of iron by this organism. When iron-organic complexes in ferrous form are the inhibitors, significant amounts of iron will be transported through the sheath and adsorb either inside or outside the cell wall. When the ferric form is the inhibitor, most of the iron will adsorb on the sheath. This difference in behavior between the iron-organic complexes of ferrous and ferric forms in adsorbing on *Sphaerotilus* can be explained by examining the hypothetical electrical double layer outside the organism.

For iron-citrate complex, each ferric iron is chelated by a citric anion so that ionic bonds are the major bonding force between the ferric cation and the citric anion. The complex is in equilibrium with its counterparts. Because citric anions are carrying negative charges, they would be repulsed by the negative charge on the surface of *Sphaerotilus*. This prevents the anion from entering the double layer while the complex with a positive charge can enter the double layer. As a result, the concentration of iron-citrate complex in the double layer will be higher than that in the bulk solution, while the concentration of the citrate anion in the double layer will be less than that in the bulk solution. In order to maintain the equilibrium in the double layer, the complex would dissociate to its counterparts, i.e., the iron-citrate decomposes to ferric cation and citrate anion. After being released from the iron-citrate complex, the ferric ion would be attracted to the surface of the organism and adsorbed on the sheath. The citrate anion would be repulsed by the negative charge on the surface of the organism and diffuse back to the bulk solution.

For the ferrous forms of the iron-organic complex, i.e., iron-cysteine and iron-gallic acid, ferrous iron is bound to its organic counterpart with a covalent bond. A covalent bond is different from an ionic bond. When the iron-organic complex is formed by a covalent bond, it does not dissociate into its counterparts in the solution. For this reason, the iron-cysteine complex is stable in the electrical double layer. However, at the surface of sheath and cell wall, the oxygen activity would be higher than that in the bulk solution due to the orientory effect of the surface. The covalent bond that connects the iron with cysteine is presumed to be broken by the oxygen reaction. The ferrous iron in the complex is oxidized to ferric form and released from its counterpart. The ferric ion is adsorbed to these surfaces and, as discussed previously, the organic counterpart diffuses back to the bulk solution.

Because *Sphaerotilus* is more vulnerable to the adsorption of iron on its cell wall than on its sheath, the ability for the iron-cysteine to transport through the electrical double layer at the surface of *Sphaerotilus* and adsorb iron on the cell wall inside the sheath makes iron-cysteine more effective in inhibiting *Sphaerotilus* than iron-citrate. This analysis suggests that iron-organic complexes in which the iron is in the ferrous form will be more effective inhibitors than ferric iron-organic complexes.

VI. CONCLUSIONS

The adsorption of iron on *Sphaerotilus* is the major mechanism which can be ascribed to the inhibition of this organism with iron. The layer of iron on the surface of the organism blocks the transport of nutrients through the sheath and cell wall and hence inhibits the growth of this organism. Among the iron compounds employed in this study, the soluble iron complexes were more effective than precipitated iron in inhibiting the *Sphaerotilus*. Iron-cysteine was the most effective of the three complexes studied.

Soluble iron complexes form a uniform layer of iron on the organism so that the inhibition effect is proportional to the iron adsorbed on the organism. The iron precipitates, on the other hand, only cover part of the surface of this organism so that their inhibition effects are much less than soluble iron complexes. Among the soluble iron complexes, ferrous forms are more effective than ferric forms in inhibiting *Sphaerotilus* because the ferrous form can diffuse through the sheath of the *Sphaerotilus* and deposit on the cell wall while most of the iron in ferric form is adsorbed on the sheath. *Sphaerotilus* is more vulnerable to the iron deposits on its cell wall than on its sheath and the adsorption of iron on the cell wall makes the ferrous iron more effective in inhibiting *Sphaerotilus*.

Several facts reveal that the mechanism for the adsorption of iron on *Sphaerotilus* is a physical-chemical process rather than a biological one. Since the surface of *Sphaerotilus* carries negative charges, the soluble iron complexes are attracted to the organism and a rapid adsorption occurs.

Because the surface of the organism provides attractive forces and sites for the iron adsorption, it is the major factor for the reaction. The concentrations of organic complexing agents and the specific iron-organic complexes also have some influence on this reaction.

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